

Steps towards challenges of prioritization

The US National Science Board has drawn necessary and welcome attention to the shortcomings of the White House's procedures for deciding what research to support.

Despite the excellence of US science, some of the research community's leaders continue to worry that a lack of coherence in policy-making leads to inefficiency and wastefulness. They point to the real and imaginary risks of weak coordination and inconsistent prioritization across the \$17 billion that US government agencies spend each year on basic research.

To cite one example, the growing community of structural biologists is increasingly reliant on neutron sources to map their structures. Most of these people are recipients of grants from the National Institutes of Health (NIH). But if any existing neutron sources are improved, or new ones built, it is the Department of Energy (DOE) that is responsible. And such funds as become available for people to use such sources will come chiefly not from DOE, but from the National Science Foundation. This confusion of responsibilities is not good for science involving neutrons.

On a more hypothetical note, there are three agencies that fly aircraft in the United States to gather meteorological research data. What happens if each agency decides to cut back its efforts, expecting the other two to pick up the slack?

Such concerns have led the National Science Board (NSB) to complain this week that existing mechanisms do not adequately ensure the sensible coordination of US science policy (see page 543). The NSB calls not just for better coordination, but for the actual prioritization of basic research disciplines against each other. "Although many scientists consider the task both undesirable and undoable," its working paper declares, "the National Science Board believes that this difficult task will become increasingly important, and must be faced over the next few years."

Independence

The NSB is well-placed to address these issues. Although it has been chiefly engaged in the management of the National Science Foundation — which accounts for less than one-sixth of the federal government's budget for basic research — the board was originally set up to advise the administration and the Congress on science policy in its broadest sense. Its members are appointed by the president on rolling six-year terms, lending it some degree of independence from any particular administration. Its chairman, Richard Zare of Stanford University, has done well to ignore the worriers inside the board's own ranks (see *Nature* 387, 220; 1997), and publish its thoughts on broad issues of science policy.

The NSB's view is that the existing system for coordinating science across the government is not working as it is supposed to. The Office of Science and Technology Policy (OSTP) — a team of forty seconded scientists and science policy experts inside the White House — is meant to do the job, reaching out to the agencies of the government through meetings of the National Science and Technology Council (NSTC). The NSTC consists of the heads of government departments and agencies. But, in practice, people such as the Secretary of Defense are too busy for this kind of thing and the NSTC never meets; most of

its work is done through nine subpanels, recently reduced to five.

The OSTP's office is understaffed and overworked — Clinton cut its size by one-third when he was first elected — and lacks executive clout. Frank von Hippel, a Princeton physicist and expert in nuclear proliferation, said it best, after a brief stint as OSTP's assistant director for national security. "Once I was inside the White House, I learned about 'turf,'" he wrote in the newsletter of the Federation of American Scientists, "and also that I had very little."

Some attribute OSTP's weakness to the leadership qualities of Jack Gibbons, the affable former physicist who has directed it since 1993. Replace Gibbons with someone with the ear of the president, they suggest, and OSTP staff will soon be able to hold their own in meetings with officials from powerful departments with real turf, such as state, defence and health.

Speculation

If the present incumbent is any guide, modern presidents of the United States appear to be absorbed in the minutiae of their daily opinion-polling data. It takes more than managerial competence for a science adviser to make an impression. Furthermore, eighteen months of speculation about Gibbons' imminent departure, unbroken by any clear endorsement of his position by President Bill Clinton or Vice President Al Gore, has been immensely harmful to the status of the OSTP in Washington. The appointment of a new leader of the OSTP, now expected early in the new year, can only improve matters.

Thus strengthened, and with the development of strong ties with the Office of Management and Budget, the OSTP would be well placed to lead the process that the NSB recommends. Prospects for better coordination clearly exist, but prioritization will be more difficult, especially in the NSB's realm of basic research. Technology programmes can be prioritized relatively easily on the basis of a country's economic and social needs: but the question of weighing fields of knowledge is altogether more problematic (cosmology against signal transduction, anyone?). It would be helpful if the NSB gave some indication of the principles on which such an exercise could be based.

Nonetheless the board is right to raise the issue publicly, if only to dispel the newly fashionable notion that funding problems for American science are about to go away. Recent bizarre discussions among some senators, congressmen and scientific societies about doubling the United States' investment in research and development should not distract anyone from the stubborn fact that budgets will remain tightly constrained.

Science advocates will be brought back to Earth with a bump in February, when Clinton is expected to propose a budget containing little or no growth for the main science agencies. Congress will then have to confront the limit that this year's balanced budget agreement has placed on that body's own natural profligacy. The community should then take its lead from the NSB, and help to answer the central question of who should have first call on limited resources. □



\$40m plant genome sequencing effort targets the best science

[WASHINGTON] The US National Science Foundation (NSF), under instructions from Congress to launch a plant genome research programme following intensive lobbying by the US Corn Growers' Association, appears to have succeeded in framing the initiative on its own, scientifically rigorous, terms.

The NSF says it will award more than \$30 million next year to teams of researchers who present the best ideas for investigating the genomes of 'economically significant' plants. Money will not be allocated in advance for the investigation of any particular crop, but will instead be sent to where NSF reviewers see the best science.

Mary Clutter, head of the NSF's directorate of biological sciences, says that the announcement reflects NSF's "ordinary way of doing business". Asked what direction the programme will take, she says: "We're going to ask the scientific community to make proposals, and find out."

The programme is being rushed into place after Senate appropriators, led by Senator Christopher Bond (Republican, Missouri), earmarked \$40 million for it in the current financial year, which ends next September (see *Nature* 388, 312; 1997). Clutter met Senate staff last week to update them on the initiative's progress, and convinced them that the NSF will make it work. "I think NSF

is embracing it as a good initiative," a staff member said afterwards.

But concerns remain. Some researchers doubt whether the programme can meet what they see as the corn growers' inflated expectations of immediate outcomes, and retain funding long enough to bear fruit.

Others worry about the principle of the NSF — an agency whose primary mission is to support science being conducted for its own sake — running errands for an influential senator from an agricultural state who happens to chair the appropriations subcommittee that funds it.

This week's announcement asks researchers for proposals that will cost up to \$3 million a year for between one and five years. The document envisages 'virtual centres' involving collaborators at many institutions, addressing questions on gene sequence and function from many angles.

Participants will be required to coordinate their work with existing international projects, such as the Japan-led effort to sequence the rice genome. No money will be available for new infrastructure or buildings.

As well as plant geneticists, Clutter hopes to see computer scientists, mathematicians and engineers involved in proposals that take new approaches to the study of plant genomes. Much of the work will use



Next in line: samples of mutated corn await genetic analysis at the University of Illinois.

sequencing methods developed by the Human Genome Project, Clutter says, "but we're always in the market for new ideas".

Researchers must submit letters of intent by 2 February and full proposals by 6 April. Grants will go out next September. Clutter says that all of the programme's \$30–\$35 million will be spent in the current year, with future spending contingent on future funds for the programme. A separate announcement will seek proposals for the accelerated sequencing of the *Arabidopsis* genome, on which the NSF will spend the rest of the \$40 million.

Peter Raven, director of the Missouri Botanical Garden in St Louis and a powerful advocate of the programme, supports the NSF's plan to fund competing teams taking distinct approaches to the study of many different plant genomes, rather than contracting people to follow an agreed masterplan.

The genomes of corn, rice, wheat and other grasses are "strikingly similar", he says, and the investigation of each will benefit the understanding of the others.

Raven is confident that the NSF can handle the sudden infusion of money into this field. "It is a huge ramp-up," he admits. "But research on plants is heavily underfunded."

NSF officials believe they have convinced the growers of corn and other commodities who sought the initiative that basic research can help to realize their objective of resilient, higher-yield crop varieties. The growers, says Clutter, "didn't really have any idea that fundamental science questions were important to them — but they now understand that".

But the growers are watching, as well as

UK institutes club together to win sponsors

[LONDON] Keen to boost their joint scientific capabilities and appeal to public and private research sponsors, nine institutes supported by Britain's Biotechnology and Biological Sciences Research Council (BBSRC) have formed a consortium known as the Bioscience Network.

The institutes, which include prominent units such as Babraham Institute near Cambridge, the John Innes Centre near Norwich, and the Roslin Institute in Scotland — home to the cloned sheep, Dolly — have a joint income of about £150 million (US\$240 million) a year, and employ about 2,000 scientists and 400 PhD students.

In a statement issued last week, the institutes say the purpose of the new network

is to provide an organization for interacting more effectively with the wide range of sponsors and customers of their research, and to "facilitate interactions between the institutes and other major contributors to the UK science base, such as the research universities".

Many in the institutes feel that the work of government-funded laboratories has been unfairly undervalued by government departments in recent years, particularly under the previous Conservative government, which was keen to find activities considered ripe for 'privatization' through the so-called — and since abandoned — Prior Options exercise.

"The institutes have been

through a particularly tough time recently," says Ben Mifflin, director of the Institute of Arable Crops Research, and chairman of the new network. Mifflin points to the results of a recent survey of citations to work published by institute scientists to rebut claims that institute researchers tend to be less productive than those in universities (see *Nature* 390, 12; 1997).

"We have been on the defensive for a long time," he says. "This is an opportunity to state our case."

The creation of the network has been welcomed by Sir Alistair Grant, the chairman of the BBSRC, as "an important step in the evolution of the research institutes". □

learning. Kellye Eversole, a Washington lobbyist for the Corn Growers' Association, says that this year's budget language deliberately gave the NSF plenty of flexibility. "No-one wanted to be real prescriptive," she says. "But if it doesn't go in the right direction, we'll be more prescriptive next year."

Eversole is perplexed by complaints from scientists that Congress imposed the initiative on the NSF. "The scientific community seems to be okay with the president setting priorities, but not with the Congress setting priorities," she says.

These complaints will come more to the fore next year, however, if the NSF's budget comes under pressure and Congress tries to protect the plant genome programme. This year, Bond was able to add the \$40 million on top of the increased funding requested by the NSF for its normal research grants.

Officials will not say what is in next year's budget, which President Bill Clinton will unveil in February. But early indications are that he may propose no increase at all in the NSF research budget, leaving programmes to scramble for funds.



Bond: pulled off bid for extra \$40 million.

Some scientists also worry that Congress will drop the programme if it does not bear early fruit. "The commitment is only for one year," says Andrew Paterson, a plant geneticist at the Texas A&M University. "That is scary, because it is difficult to quickly make the kind of high-visibility findings which the Congress will recognize as a basis for giving us more support." Senate staff say this concern is misplaced, and that Bond is with the project for the long haul.

"We're talking about a major effort," says Clutter. "What the NSF is doing is jump-starting something that will set the stage for agriculture of the 21st century. But it isn't something that NSF will be doing for ever."

Other agencies, particularly the US Department of Agriculture, are expected to be involved in the initiative. A multi-agency task force, chaired by Ron Phillips, USDA's chief scientist, is completing a report on what their effort will look like.

The Senate has proposed an immediate injection of an extra \$780 million over five years into five important areas of agricultural research, of which plant genetics is one. This effort ran into trouble when the House of Representatives went into recess last month without passing a companion bill.

But there is a good chance that USDA funds will be made available for plant genome work. That would mollify those at the land grant colleges who do most USDA research and worry that they will be overlooked by the NSF.

Colin Macilwain

Greenwich observatory's fate hangs in the balance

[LONDON] The fate of the Royal Greenwich Observatory in Cambridge will be decided tomorrow (12 December) when Britain's main astronomy research funding agency decides whether to throw the centre a life-line or to carry out its original plan to close its research activities.

The Particle Physics and Astronomy Research Council (PPARC) announced in July, after an intense debate, that it had decided to close the Cambridge site and merge some of its activities with those of the Royal Observatory in Edinburgh to create an Astronomy Technology Centre (see *Nature* **388**, 105; 1997). The £4 million (US\$7.2 million) annual savings will be used to support Britain's university astronomy sector.

But the decision to close the Cambridge centre after the merger has been fiercely opposed by the observatory's management, which has put together its own proposal to convert it into a private company. The managers believe they can use the observatory's world-famous name to sell its expertise in telescope design and instrumentation.

Under a business plan presented to PPARC, the observatory would remain in Cambridge, to which it moved less than ten years ago from its previous site at Herstmonceux Castle in Sussex, where it had been located since moving from its original site in London. Initially, half of its contract work would come from PPARC. The rest would be divided between different UK and, eventually, foreign government agencies.

Neil Parker, the observatory's assistant director, says the management is confident its plan makes financial sense, and will enable the observatory to continue as a research organization. But at the beginning of this week it remained clear that the decision will not be an easy one for PPARC.

When they meet tomorrow, members of PPARC's council will examine reports from separate committees that have examined the observatory management's proposals in detail. A committee of senior astronomers has reported on the proposed research plans. And the business plan has been reviewed by an internal audit committee, as well as by the accountancy company Touche Deloitte.

PPARC will not comment on the reports' conclusions. But the reports — and tomorrow's discussion — are almost certainly expected to address three important concerns. One is the question of start-up capital for the new company. The observatory is believed to have asked PPARC to provide £1 million to help pay salaries while order books remain thin. There is likely to be considerable debate as to whether the research



Fighting for life: observatory officials want to keep the Cambridge centre (above) open.

council can afford such a contribution.

The other concerns relate to the implications for PPARC, and for any contracts it funds, if the company proves unable to sustain itself, and to the implications for the University of Cambridge. The observatory's accommodation is owned by the research council on land leased by the university.

The university likes to maintain a distinction between academic facilities, based within the university, and private enterprise, based in the university's science park. It has not yet indicated whether it will allow a private observatory to continue operating from the university's 'academic' sector. In a recent statement, the vice-chancellor, Alec Broers, said the university was in no position to offer financial assistance to the observatory.

Relations between the university's Institute of Astronomy and the observatory have never been close — something Parker says the new company will try to remedy.

If PPARC were to refuse the observatory management's plans, it might revert to the 'fall-back position' of returning the observatory's name to its original home in Greenwich, now part of the National Maritime Museum (see *Nature* **388**, 705; 1997).

The museum's officials are known to be keen on this idea. They want to set up a centre for the public understanding of science. They believe that bringing the name back to Greenwich would boost public interest, particularly in the run up to the millennium.

But, as far as the observatory's managers are concerned, that is the least attractive option. They emphasize that the observatory is at the forefront of research in telescope instrumentation and design, and are tired of the constant references to its past. "The Royal Greenwich Observatory is not a museum," says Parker.

Ehsan Masood

UK accused over risk of CJD in plasma...

[PARIS] Foot-dragging by the British government is exposing haemophiliacs to an avoidable risk of infection with the new variant of Creutzfeldt-Jakob disease (vCJD) from contaminated blood products. That was the warning issued last week by the organization that represents the directors of 109 centres that treat haemophilia and other complex blood diseases.

The government has acknowledged that blood products pose a potential risk of transmission. But it has so far limited action to commissioning a detailed risk assessment of the problem, and asking the National Blood Authority to consider removing lymphocytes from the blood, given that the causative agent of vCJD may occur in these and other lymphoreticular tissues (see *Nature* 390, 105; 1997).

In a letter to *The Lancet*, however, the UK Haemophilia Centre Directors' Organization says the potential risk is already obvious. It calls for immediate action to protect haemophiliacs by switching them from blood products prepared from UK plasma

supplies to either recombinant alternatives or products prepared from donor plasma collected in countries with no recorded cases of bovine spongiform encephalopathy (BSE) or vCJD.

Christopher Ludlam, the chairman of the organization, says the letter is an attempt to draw attention to what he characterizes as a ludicrous situation — that, whereas the European Agency for the Evaluation of Medicinal Products' (EMA) Committee for Proprietary Medicinal Products has recommended that plasma from donors with vCJD should not be used, the absence of a diagnostic test for vCJD means it is impossible to screen donors.

Given the high level of exposure of the UK population to BSE during the 1980s, and the long incubation period of spongiform encephalopathies, many seemingly healthy donors may be harbouring the disease.

The potential risk for haemophiliacs is much higher than for other transfused patients, as they receive multiple transfusions of products prepared from pools of



plasma derived from 20,000 to 50,000 individual donations. "There is therefore obviously a much increased risk of getting at least one infected donation in the pool, and one batch of factor VIII may go to 50 haemophiliacs," says Ludlam.

Most haemophiliacs in many countries — including France and Ireland — already exclusively use recombinant factor VIII on the grounds that it is completely safe. But the UK Department of Health has rejected the recommendation of the directors of haemophilia centres that Britain should eliminate all risk of vCJD transmission to haemophiliacs by simply following suit. The department argues that it is up to local authorities to decide. In a statement released last week the health department described the directors' alert as worrying patients unnecessarily.

John Purves, a spokesman for the EMA, says the agency intends to convene an expert meeting on the problem early next year, which is likely to yield new recommendations. The arguments in the letter to *The Lancet* will be "taken into account", he says.

Local authorities are reluctant to switch to recombinant products because they are more expensive. But the difference in price results largely from the fact that recombinant products are subject to UK value-added tax whereas plasma-derived products are not.

"The net cost of the UK going over to recombinant factor VIII would be £25 million (US\$42 million)," estimates Ludlam. He says this is a pittance compared with the £3 billion spent on BSE-related subsidies.

Ludlam's frustration is shared by many researchers who feel that the risk from blood products is receiving insufficient attention. "I took part in the discussion in the early 1980s over HIV and blood, and I have that awful sinking feeling all over again; the issues are so similar," says Ludlam. "All we need is one haemophiliac to get vCJD and all blood products will be removed from the market."

Declan Butler

...and latest BSE beef ban 'comes too late'

[PARIS] Repeated assurances by the UK government that British meat is now safe to eat came to an abrupt end last week when it banned the sale of all cuts of beef on the bone on the grounds of new scientific evidence showing infectivity in bone tissues. The ban includes T-bone steaks, ribs and oxtail.

The government acknowledged last March that the agent that causes bovine spongiform encephalopathy (BSE) might pass to humans (see *Nature* 380, 273; 1995). But the official line has been that a 1990 ban on consumption of specified bovine offals has been sufficient to protect the population, and that reported cases of the new variant of Creutzfeldt-Jakob disease would have been caused by consumption of such tissues before that date.

But, according to the government's Spongiform Encephalopathy Advisory Committee (SEAC), research by the Ministry of Agriculture, Fisheries and Food in which cattle were fed BSE-

contaminated tissues has also revealed infectivity in dorsal root ganglia, trigeminal ganglia and bone marrow.

The ban on beef on the bone was hastily announced by Jack Cunningham, the agriculture minister, after SEAC's advice to the government was leaked to the press. But it is likely to have little impact on public health. According to SEAC's own estimates, as BSE is now dying out in British herds, only about six of the 2.2 million cattle expected to be slaughtered next year are likely to be infective.

Stephen Dealler, a UK BSE researcher, is one of many scientists who argue that the research results are largely "of historical interest, in that they tell us what we have been eating". They argue that the latest ban confirms that the government decision-making process in the BSE crisis has focused on responding retrospectively to firm scientific results rather than taking sufficient precautions against anticipated potential risks —

such as that to haemophiliacs (see above).

This issue is likely to come under scrutiny as part of the inquiry into the former Conservative government's handling of the BSE crisis which the new Labour government announced last week. One question will be the basis of previous assurances on the safety of beef on the bone.

Mark Savay, a French BSE researcher, points out that the choice of cattle tissues originally banned in the United Kingdom and Europe was based not on direct experimental evidence of their infectivity, but on the known distribution of scrapie infectivity in sheep.

Savay points out that the European Commission banned exports of UK beef on the bone in 1990, except where it originated from herds where BSE had not been reported for two years. "What is extraordinary is that the same logic should have been applied to UK consumers but it wasn't," he says.

D. B.

Geologist fails to overturn Creationist judgement

[SYDNEY] Ian Plimer, the Australian professor of geology who has fought a five-year court battle against a Christian preacher's claims to have found 'scientific' evidence for Noah's Ark in Turkey, has lost his bid to overturn a judgement made against him last June.

Three Federal Court judges found unanimously last Friday (5 December) that the original judge, Ronald Sackville, was correct in dismissing Plimer's civil action against preacher Allen Roberts (see *Nature* 389, 323; 1997). The presiding judge, John Davies, acknowledged that Roberts had made "misleading statements" in his lectures. But he concluded that such conduct was not an aspect of "trade or commerce", which was the basis claimed by Plimer for his charges against Roberts.

Outside the court, Roberts said Plimer had "sought to stifle our voice", and declared "a victory for freedom of belief and expression". Plimer admitted his "pockets now rattle after the appeal", and said he will file for bankruptcy.

Despite the setback, Plimer and his solicitor say they may appeal further to the High Court of Australia over the lack of protection for consumers—the basis of their challenge that creationism was being presented as science—implied by the Federal Court's judgement.

While the High Court may not give leave for an appeal, another court confrontation appears certain, as a suit for defamation brought by Roberts against Plimer was deferred until after the trade practices case. Plimer says he will immediately seek a trial date, believing he can plead evidence and issues of broader scope than were allowed under trade law.

Meanwhile, the Creation Science Foundation, a Brisbane-based evangelical group apparently unconnected with Roberts' group, has changed its name to 'Answers in Genesis'. The foundation, which teaches 'creation science' and campaigns for its inclusion in school curricula, claims scientific authority primarily through a staff member who holds a PhD in geology, and its director, who is a medical practitioner.

Barry Williams, executive officer of the Australian Skeptics, argues that the name change can be seen as a response to the threat of similar legal actions to Plimer's, and labels it as "a triumph for the side of reason and science". According to Williams, the foundation "has publicly confessed that what they do is not science, a very useful concession to our case".

Peter Pockley



Rural calm? The JET fusion plant, source of a long-running employment disagreement.

Dispute blocks agreement on Europe's fusion funds

[MUNICH] British scientists working at the Joint European Torus (JET) fusion facility in Oxfordshire, England, have overwhelmingly rejected a settlement proposed by the European Parliament which could have resolved a fifteen-year labour dispute.

The dispute centres on the fact that the scientists are hired on contracts issued by the UK Atomic Energy Authority (UKAEA), whose terms are less advantageous than the European Atomic Energy Community (Euratom) contracts enjoyed by their non-British colleagues. Two-thirds of the JET scientists are hired by UKAEA.

The failure to reach a resolution has already led to the parliament blocking the 1998 budget for the entire European Union fusion programme in an attempt to force a negotiated settlement. The parliament's budget committee will decide next week how much of the fusion budget to release, and whether it wishes to continue trying to help resolve the dispute, which could affect any future extension of the JET programme beyond 1999.

The European Court of Justice ruled in 1987 that, although JET's dual employment system was discriminatory, the differences in contracts were justified as non-Britons had to live abroad and, at the time, lacked the prospect of a guaranteed job when the JET programme ends. After this ruling, the JET council gave the British scientists a one-off *ex gratia* payment of ECU2 million (US\$2.2 million) in compensation.

But in a second ruling last December, the court found that conditions had changed, as much of UKAEA had since been privatized, and the scaled-down organization was no longer capable of absorbing the British scientists when the JET joint undertaking comes to an end.

At the same time, the European Commission started actively seeking new positions

for Euratom JET employees. This time the court ruled in favour of the UKAEA workers.

The commission, which funds 80 per cent of JET's costs, the JET council, the UKAEA and the British JET scientists entered negotiations on how the scientists should be compensated following the new ruling, but these broke down in early summer.

Employers were unable to meet the demands of the British scientists for either transfer to Euratom contracts, or full financial compensation, which would have totalled ECU21 million after tax, for the three-year JET programme. As the settlement would probably be subject to full taxation, this demand would actually have cost the programme around ECU45 million.

In an unusual move, the parliament decided to act as mediator in the negotiations. In its October plenary session, it blocked the 1998 fusion research budget in a bid to force the parties back to the negotiating table.

The settlement proposed by the parliament—but subsequently rejected by the British JET scientists—included transferring 25 Euratom JET positions to the general fusion programme, making them open to competition from British JET researchers, along with a total *ex gratia* payment of ECU9 million to the UKAEA staff in compensation.

Martin Keilhacker, director of the JET programme, says that the JET council, which met after last week's ballot, would have been prepared to accept the parliament's proposal as a final settlement of the dispute, "despite the burden this would have placed on JET's [very stretched] budget".

The parliament is expected to lift its block on the general fusion budget at its plenary session next week, while possibly holding back a reserve to cover future compensation payments. It is unlikely to continue to act as mediator in the dispute

Alison Abbott

Science board seeks priority agreement

[WASHINGTON] The US National Science Board (NSB) has harshly criticized the way that science policy is established in the US government, and called for a dialogue between interested parties to establish procedures for setting scientific priorities and coordinating science-related activities across government.

According to several officials, the recommendations of the NSB — a group of senior mainly academic scientists appointed by the president, primarily to run the National Science Foundation — amount to a rebuke for the White House Office of Science and Technology Policy (OSTP), which is responsible for such coordination.

The board's working paper, *Government Funding for Scientific Research*, accepts that the White House successfully coordinates science policy "in areas of special national interest", such as global climate change and clean-car technology. But beyond such "special areas", it says, coordination depends on a series of *ad hoc* arrangements "and the memories of Congressional committees".

The board calls on all those interested to collaborate in an effort to work out how to identify scientific priorities and implement the coordination of science policy. "The board offers its assistance on this critical task in any way that the president and the Congress would find helpful," the 15-page paper concludes.

Richard Zare, a professor of chemistry at Stanford University and chair of the board, who initiated the 18-month effort to produce the working paper (see *Nature* **382**, 286; 1996), says that the objective is "to get the attention of other stakeholders and get a dialogue going".

The paper does not say who should fix priorities, how they should be set or how they should be implemented. Zare says that is because these things "need to be done with all of the interested parties, together".

One option would be for the National Science and Technology Council (NSTC), the committee of cabinet members supposed to coordinate science between agencies in the government, to undertake the task. "There was discussion of that possibility, and of many others," says Zare. "But we decided it wasn't clear at present who should do it."

Zare concedes that his effort to get the board to advise the government on science policy beyond the confines of the National Science Foundation (NSF) has proved more difficult than he expected. "It has been a very tough issue," he says.

"But it remains my view — and I think the view of the board too — that we should deal with issues bigger than the NSF." The foundation accounts for just over \$2 billion of the \$17 billion that the US government spends



Zare: keen to broaden dialogue and improve policy coordination.

on basic scientific research each year.

The NSB endorses a suggestion made two years ago by a panel of the National Research Council, chaired by Frank Press, the former president of the National Academy of Sciences, that Congress should review the research budget as a single entity each year, before it is divided up for consideration by the 13 appropriations subcommittees.

But it ignores — and implicitly rejects — Press's suggestion that the research and development budget be redefined to exclude billions of dollars of essentially non-innovative development work, particularly in the Department of Defense.

The board's paper may focus criticism of both OSTP and the NSTC, according to some officials who saw it last week. OSTP, which has about 40 staff in the White House,

is supposed to advise the president and implement his scientific priorities, primarily through meetings of NSTC subcommittees, which bring together senior officials from different government agencies.

The effectiveness of both organizations has been in question for some time. But recent criticism has centred on the position of Jack Gibbons, the director of OSTP. One senior science-policy expert, who declined to be identified, described OSTP as "in meltdown" at present, and congressional staff of both parties are privately critical of Gibbons's performance.

Gibbons declined to be interviewed on the board's findings, but issued a statement describing the paper as "a thoughtful, insightful reflection of the diverse and mature perspectives of the NSB". He added that it also accurately reflects the complexity of decision-making on the allocation of public funding, especially in times of unrelenting budget pressure. "I am delighted to see NSB take more direct interest in the broad issues of science policy," he said. **Colin Macilwain**

US wins access to Framework programme

[WASHINGTON] The United States and the European Union (EU) have signed a collaborative agreement on science and technology which, according to officials on both sides of the Atlantic, will enable US scientists to participate in key elements of the EU's Framework research programme for the first time.

The agreement, which was signed in Washington DC last week, had been sought by US agencies — especially the National Science Foundation — which have been keen to get involved in parts of the \$4-billion-a-year Framework programme, particularly biotechnology and materials science, which have previously been closed to US participation.

Virtually all the natural sciences and engineering fields will be covered, and in each the agreement will offer opportunities for reciprocal participation in research activities. But, at the behest of the United States, the deal rules out biotechnology research that might yield new varieties of plants or animals until all the European countries have passed legislation protecting such inventions.

Also, despite two years of negotiations, the EU has failed to gain concessions that would win corresponding access for European participants in US programmes. US officials argue that arrangements for foreign access to such programmes — which vary widely — are determined by the laws governing the individual programmes, and

could not be revised through this agreement.

"The Europeans wanted a completely open agreement, as they have with Canada," said one European diplomat in Washington. "But they ended up with something much weaker". Basic research programmes in the United States are often open to foreign participation, but industrial research programmes are usually closed to foreign-owned companies, and this will continue to be the case.

The agreement signed last week is a so-called 'framework' deal, defining a structure for intellectual property rights and related issues to act as the basis for future, specific deals between US agencies and the EU. Officials say it will take a year or two for such deals to be made and for collaborative work to start. "This is where the real work begins," said one US negotiator.

The two parties originally decided to negotiate the agreement at a summit meeting in Madrid two years ago (see *Nature* **379**, 3; 1996). It was signed last week by Strobe Talbot, US deputy secretary of state, and Jacques Poos of Luxembourg, president of the Council for the European Union, at a White House ceremony.

The deal "will serve as a broad framework for scientists to work together and establish new projects," said Poos. The agreement will initially stand for five years, but may then be extended following a review in the final year. **C.M.**

Asia/Pacific 'bionetwork' comes to life

[TOKYO] Only six months after it was first publicly discussed, a network of life scientists in the Asia-Pacific region that aims to boost biomedical research and biotechnology in the area has begun to take shape. The network will be modelled on the European Molecular Biology Organization (EMBO).

Meeting in Shanghai, China, at the end of last month, representatives of the founding members of the International Molecular Biology Network for Asia and the Pacific Rim (IMBN) agreed on rules for membership and a draft constitution. They also elected key officers, and pulled together sufficient seed funding to begin operations next year.

The concept of the network was discussed at a meeting of scientists from throughout the region in June (see *Nature*, 388, 3; 1997) and was endorsed by science officials of the Asia-Pacific Economic Cooperation (APEC) forum in Singapore in October.

Ken-ichi Arai of Tokyo University's Institute of Medical Science has been elected chairman of a task force that will draw up an initial membership of about 200 scientists from the region by next spring and set up a governing council. Jeongbin Yim, director of the Institute for Molecular Biology and Genetics at Seoul National University in Korea, and Louis Lim of the Institute of Molecular and Cell Biology in Singapore,

will both act as vice-chairmen.

The executive director and coordinator of the network's secretariat is Gurinder Shahi, a founding father of the International Vaccine Institute in Seoul (see *Nature* 389, 655; 1997). He will be based in Singapore, but expects the network to have 'virtual' offices throughout the region, which he will coordinate. As in the case of EMBO, members will be selected on the basis of scientific 'excellence'; EMBO is expected to help by screening potential members, says Shahi.

The task force set approximate membership quotas for each country or economy in the area. Japan is expected to have up to about 40 members, Australia, China, Chinese Taipei (Taiwan), and Korea up to about 20 each, Hong Kong, India, New Zealand and Singapore up to about 10 each, and Indonesia, Malaysia, the Philippines and Thailand up to about 5 each.

But the task force also wants membership to be open to individuals from other countries in Asia and the Pacific Rim, including, potentially, the United States, Canada, Russia and Latin American countries in APEC.

A governing council will be elected from among the founding members, probably with one representative from each country/economy in the region, but possibly with one extra representative for those with large

numbers of members. "Such an approach will help ensure broad representation while enabling more scientists from economies with a concentration of excellence to serve IMBN," says Shahi.

The first meeting of the governing council will be held in June in Pohang, South Korea, in association with IMBN's first conference, to be held that month in Seoul. A second phase of membership development is planned after June with membership on two levels — 'members' who will be able to vote and serve as members of the governing council, and 'associates', those committed to the idea of IMBN but not yet meeting the organization's criteria of excellence.

Several participating institutions have agreed to provide seed financial support to kick-start IMBN activities, and funding commitments totalling more than US\$300,000 have already been made by institutions, government and industry in the region.

IMBN plans to support short-term fellowships for scientists and graduate students to visit centres of excellence for collaborative research and/or training. It will also sponsor courses and workshops and provide small grants for collaborative research involving scientists from two or more economies.

David Swinbanks

Taiwan looks to the skies for a boost to its basic research

[TOKYO] Taiwanese astronomers have won approval for a small but ambitious international project to carry out the first survey of the outer regions of the Solar System to look for comets and small objects. The Taiwan-American Occultation Survey (TAOS), a joint two-year project with the Lawrence Livermore National Laboratory's Institute of Geophysical and Planetary Physics (IGPP) in the United States, will lead to the construction of the first observatory in Taiwan to carry out international research.

Astronomers at Taiwan's Academia Sinica and National Central University will work alongside researchers from the Lawrence Livermore National Laboratory in California, who carried out the Massive Compact Halo Object (MACHO) project.

The astronomers plan to use three automated 0.5-metre optical telescopes, stationed at a height of 2,800 metres and located within 7 km of each other in the Jade Mountains in Taiwan, to survey the Kuiper Belt. They will conduct a census of objects down to 3 km in size, and will also survey the inner Oort Cloud.

Two-thirds of the \$500,000 funding for the new TAOS hardware comes from organizations in Taiwan including Academia



High hopes: the Jade Mountains will host three new telescopes.

Sinica, the National Science Council, and the Ministry of Education, with the remainder coming from the US Department of Energy. The telescopes will be operational within two years.

Basic research and astronomy are growing rapidly in Taiwan, fuelled by increasing numbers of researchers returning to Taiwan from the United States (see *Nature* 383, 12; 1996). "This is how the electronics industries developed in Taiwan 10–15 years ago when experienced engineers and managers were brought to Taiwan," says K. Y. Lo, the director of the Institute of Astronomy and Astrophysics at Academia Sinica, who himself recently returned to Taiwan from the University of Illinois.

An influential panel of Chinese astronomers and astrophysicists based in the United States and Canada, led by Frank Shu of the University of California at Berkeley, have played an important role in planning

projects and building momentum for the Taiwanese study. Five years ago the panel was instrumental in creating the Astronomy and Astrophysics Institute at Academia Sinica, Taiwan's leading government research institute. It was also involved in the decision by Academia Sinica last June to expand, through a partnership with its new institute, the Smithsonian Astrophysical Observatory Sub-Millimeter-wave Array (SMA), increasing the number of telescopes on Mauna Kea in Hawaii from six to eight.

It is hoped that the high technology used in astronomy will stimulate Taiwanese industry. "Basic research needs to develop to stimulate further high-tech development," Lo argues. There is already some spin-off from these projects: two of the SMA telescopes to be stationed in Hawaii are being built in Taiwan, and structural carbon-fibre reinforced plastic tubes for all eight telescopes are being built by a bicycle-frame manufacturer in Taiwan.

Astronomers in Taiwan hope that SMA and TAOS will increase the discipline's visibility in Taiwan by attracting students and encouraging university physics departments to expand their astronomy and astrophysics programmes.

Richard Nathan

Australia lifts budget threats to public research

[CANBERRA] A long-awaited statement on industry policy by Australia's Coalition government has relieved the Cooperative Research Centres (CRCs) from closure, and dropped a proposal that national research agencies and universities should substantially increase their external earnings.

In addition, recent cuts in government support for industrial research and development have been partially restored, while the board responsible for that programme has escaped abolition.

A major review by the businessman David Mortimer had proposed that funding to the 65 CRCs be cut from an annual A\$146 million (US\$108 million) to A\$20 million. But the association of CRCs predicted this would lead to the "devastation" of a programme that combines the work of universities, research agencies and industries in targeted projects. Chief scientist John Stocker attacked Mortimer's recommendation.

Mortimer also wanted the research agencies to increase their external earnings, the largest, the Commonwealth Scientific and Industrial Research Organization (CSIRO), "from 30 to 50 per cent". But the CSIRO's chief executive, Malcolm McIntosh, said Mortimer "used the wrong base" and the change "would cripple strategic research and destroy CSIRO" (see *Nature* 388, 509 & 819; 1997.)

Mortimer proposed a billion-dollar investment fund to improve industrial competitiveness. This would have been created partly from cuts to research, and the government's rejection of this idea has allowed the reprieve for science. But some of Mortimer's recommendations have been accepted, notably the setting of an economic growth target which the government has pitched at "over 4 per cent over the decade to 2010".

The package contains several measures costing A\$1.2 billion over five years. Direct funding of business R&D through a grant scheme called START has been extended by A\$139 million annually to a total of A\$739 million over four years. The government did not respond to the pleas of industrialists and scientists for restoration of a 150 per cent tax concession for in-house company R&D. The rate is left at 125 per cent.

The Labor Opposition spokesman on industry, Simon Crean, calculates that funding cuts to R&D have been A\$2 billion over the 1996 and 1997 budgets, only a quarter of which have now been restored.

But the industry, science and tourism minister, John Moore, says the START scheme targets "quality R&D" and the overall policy will "build vibrant, internationally competitive industries".

Peter Pockley

Kyoto meeting bridges gaps, though differences remain

[KYOTO] Prospects for a legally binding treaty to reduce greenhouse gas emissions edged a step closer at the beginning of this week as the 10-day United Nations climate conference in Kyoto moved towards its climax on Wednesday (10 December).

But the final outcome remained uncertain, depending on how far the United States was prepared to take a promise to be "flexible" made on Monday by Vice-President Al Gore.

Following eight days of tense discussions, the chairman of the conference, Raul Estrada Ouyela, Argentina's ambassador to China, set out a final consensus proposal, which he described as a "take it or leave it document". The document proposed a treaty in which developed countries would reduce their greenhouse gas emissions by an average 5 per cent from 1990 levels between 2006 and 2010.

This proposal mirrored that of Japan. It was lower than the European Union's proposed 15 per cent reduction from 1990 levels by 2010, but higher than the US proposal to

stabilize emissions at 1990 levels before 2012.

Estrada proposed that the treaty should apply to three gases: carbon dioxide, methane and nitrous oxide. A separate protocol, to be negotiated at the next conference of the climate convention, would apply to other greenhouse gases.

The 5 per cent reduction figure is an average for all 39 developed countries that are parties to the convention. It was calculated by a process known as 'differentiation' — the idea that different countries can reduce or increase their emissions according to their circumstances. Most developed countries would have to reduce emissions by 8 per cent, except the United States, Canada and Russia, which are allowed a 5 per cent reduction, and Japan, which is allowed a 4.5 per cent reduction.

In contrast, Australia — which continues to oppose legally binding emissions reduction targets — would be allowed to increase its emissions by 5 per cent. Norway would be permitted to increase emissions by the same amount, and Iceland by 10 per cent.

In a concession to countries such as Canada and New Zealand with large forestry resources, a country's emissions inventory would include both carbon dioxide from fossil-fuel sources and carbon released into the atmosphere from deforestation. This carbon 'subtotal' would then be reduced by the amount of carbon removed from the atmosphere through planting new trees. But a target date for the inclusion of carbon from forests has been deferred until scientists find a more accurate way of measuring the release and uptake of this carbon.

Ehsan Masood



Cold sweat: ice penguins melt in the sun as they await a result from the climate conference hall.

CERN deal 'historic and path-breaking'

[WASHINGTON] US and European officials have signed the agreement under which the United States will contribute \$531 million towards the construction of the Large Hadron Collider at the European Laboratory for Particle Physics (CERN) in Geneva, Switzerland.

"This is the first time we've made an investment like this in a facility outside the United States," said Federico Peña, the US energy secretary, after the signing ceremony at the White House this week. Peña described the deal as "historic and path-breaking".

The US contribution will consist of \$200 million for the construction of the collider and \$250 million for its two detectors, ATLAS and CMS, from the Department of Energy, and a further \$81 million for the detectors from the National Science Foundation (see *Nature* 385, 103; 1997).

Officials paid warm tribute to Sidney Drell, deputy director of Stanford Linear Accelerator Center. In 1994, Drell chaired a panel that identified participation in the Large Hadron Collider project as a top priority for US high-energy physics after Congress had cancelled the Superconducting Super Collider project in Texas, and explained how it could be paid for without disrupting other physics research.

Christopher Llewellyn Smith, director general of CERN, said that the \$6 billion project will be completed in 2005, three years earlier than would otherwise have been possible, thanks to the involvement of the United States and Japan. US delegates will attend a meeting of the CERN council next week as observers and will hear that the programme is on time, on budget and "in excellent technical shape".

Colin Macilwain

Austria backs neutron facility — despite negative assessment

[STRASBOURG] The Austrian ministry of research has agreed to provide ÖS1 billion (US\$80 million) to support the development of a new neutron facility, known as Austron — despite a negative assessment of the proposal by an independent panel of experts organized by the European Science Foundation. The panel had criticized the proposal for its superficial costing and strategic analysis of the European need for a neutron scattering source in this location.

The ministry has given the go-ahead for Austrian scientists to develop further the scientific case for Austron, but says the money promised for its realization must be matched by an additional ÖS2 billion from external sources, such as the European Commission. After years of indecision, the government appears keen to proceed with the project primarily because it has no large-scale facility to attract foreign scientists.

Veterinary surgeons 'should boost research'

[LONDON] The report of an inquiry set up by the Royal College of Veterinary Surgeons and published by the Wellcome Trust in London this week says that more research is needed into the health and welfare of animals. It also says that the veterinary profession should make a fuller contribution to research than it does at present.

The committee, chaired by Lord Selborne, a former chairman of the Agricultural and Food Research Council, says that clinical teaching staff in veterinary schools are often too busy to make a proper contribution to research, and that new initiatives are needed to allow them to do so. It says that closer links are needed between the various bodies that carry out research into animal health and welfare.

Italy selects finalists for satellite mission

[MUNICH] In an atmosphere of rare optimism, Italian space scientists last week selected six proposals for small satellite missions to undergo feasibility studies.

The winning proposals — between them covering X-ray astronomy, gamma-ray astronomy, geophysics, telecommunications science and fundamental physics — will share IL1.5 billion (US\$0.86 million) to complete their feasibility studies before the final selection in December 1998.

The chosen mission is expected to fly in 2002. The Italian Space Agency, which appears to be entering a new phase of

stability after years of chaos, is funding the initiative — the first of a regular series of national small satellite missions unique in Europe.

Japan budget will back global warming moves

[TOKYO] The Japanese Prime Minister, Ryutaro Hashimoto, announced last week that the government is to introduce special financial measures aimed at preventing global warming in the next annual budget, to be published at the end of December. Research in environmental protection will be given a share of the ¥15 billion (US\$116 million) special budget set aside for economic structural reform.

Investment will also be made in other areas in science and technology and telecommunications. Hashimoto's announcement included a plan to set up a new research institute on global warming.

Europe to improve animal welfare

[PARIS] Sixteen European countries and 12 international organizations last week agreed to cooperate to find ways of improving the welfare of laboratory animals, and primates in particular. Under the terms of a 'declaration of intent' signed under the auspices of the Council of Europe, they agreed, for example, to choose means and routes of transport to avoid delays and to minimize 'stress and suffering', to acquire animals only from establishments with high quality housing, care and welfare facilities, and to supply the public with 'accurate information on the use of animals'.

The signatories also agreed to encourage measures to phase out the use of animals caught in the wild, and to limit use to those that are purpose-bred.

Microsoft plans software centre in India

[NEW DELHI] Microsoft Corporation plans to set up a software development centre in India, its first fully fledged product development centre outside the United States. According to the company's vice-president, Orlando Ayala, the Indian centre could be used to develop a new kind of software unrelated to earlier products such as Windows and MS-DOS.

Microsoft has been investing in India for three years. Dewang Mehta, executive director of the National Association of Software and Service Companies, says the company's decision to set up the centre in India shows that its founder, Bill Gates, who visited India early this year, is confident that the country will emerge as a software superpower in the next century.

Myriad receives patent for BRCA1 mutations

[WASHINGTON] Myriad Genetics last week announced that it had been awarded a patent on 47 different mutations in the *BRCA1* gene, abnormalities in which substantially heighten risk of breast and ovarian cancer. The award "establishes a broad and substantial proprietary foundation for the company's products," said Peter Meldrum, Myriad's president and chief executive officer. On the same day, Myriad, a genomics and genetic testing company based in Salt Lake City, Utah, announced that it is suing Oncormed, a developer of gene-based diagnostic services based in Gaithersburg, Maryland, for infringement of the new patent.

Administrative staff 'too isolated' at NIH

[WASHINGTON] A management consultant firm hired to examine the business practices of the US National Institutes of Health (NIH) has concluded that the vast agency generally runs itself well in scientific areas, but that when it comes to purely administrative functions it "becomes more and more recognizable as a normal federal bureaucracy, and less like world-class organizations".

Arthur Andersen, which studied NIH's administrative practices for seven months, recommends in a report presented to its director, Harold Varmus, last week that scientists should work more closely with NIH administrators. "The perception that the administrative staff is outside the mainstream of NIH's work undermines administrative morale and reinforces inefficiencies rather than addressing them," the report says.

Art sale restores funds to heart foundation

[BOSTON] Works from a substantial art collection amassed by the paediatric cardiologist Bernardo Nadal-Ginard, and now owned by the Children's Heart Foundation — a group practice he founded — have been sold to defray the \$6.5 million that Nadal-Ginard has been ordered by a federal judge to pay the foundation.

Formerly on the staff of the Harvard Medical School and Children's Hospital in Boston, Nadal-Ginard began serving a one-year prison sentence in March following a conviction for embezzlement (see *Nature* 375, 438; 1997). About 200 works have been sold from the 250-piece collection in auctions held this year by Sotheby's in New York and London. The third and final sale took place in New York last month. In total the auctions have netted more than \$5 million.

Red grouse and their predators

Sir — We read with interest articles by May¹ and Mead² on the persecution of hen harriers and the impact of raptor predation on grouse-shooting bags. Some of Mead's comments, however, are misleading³.

Mead suggests that the economic effects of harriers on grouse shooting are minimal. Unfortunately, that is not always the case. We recently completed six years of research on harrier and peregrine predation on grouse at Langholm in southwest Scotland³. Raptors had bred freely on this moor since 1990, and female harrier numbers increased from two to twenty between 1992 and 1997. Peregrine numbers increased from three to six pairs.

When raptor numbers were high, they removed 30% of breeding grouse in April and May and harriers removed 37% of the grouse chicks between June and August. Most of these losses appeared to be additional to other mortality, and we estimated that they reduced post-breeding numbers of grouse by 50%.

Historically, grouse bags at Langholm have shown a six-year cycle, peaking last in 1990, with 4,038 grouse shot (Fig. 1). Since 1990, grouse bags have declined, with 51 birds shot in 1997.

In contrast, grouse bags on two nearby moors, with low raptor densities, having previously fluctuated in synchrony with Langholm moor, increased to high levels in 1997.

Increased predation by raptors at Langholm was considered the most likely cause for low grouse bags. Grouse management at Langholm cost £99,500 (US\$168,000) in 1997 and, with grouse shooting producing £40 per bird, a bag of 2,487 grouse was required to balance costs.

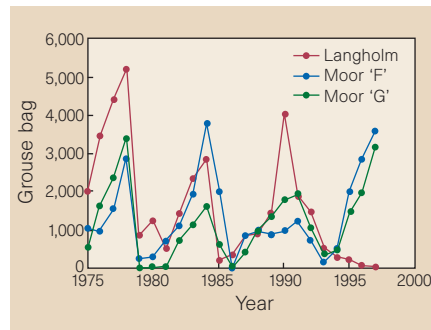


Figure 1 Numbers of red grouse shot on Langholm moor during 1975–97 in comparison with numbers shot on nearby moors F and G during the same period. Grouse bags on all three moors fluctuated in synchrony from 1975 to 1993. After 1993, grouse bags on moors F and G increased while bags on Langholm moor continued to decline. Harrier and peregrine numbers on Langholm moor increased between 1992 and 1997 whereas the numbers of these raptors on moors F and G remained low.

Clearly, if bags remain low the economic cost will be considerable.

Mead² suggests that more red grouse are killed on deer fences than are taken by harriers, citing work in Highland Scotland⁴.

There are several flaws in this argument. First, deer fences are uncommon in red grouse range outside the Highlands, and indeed on many Highland moors. For example, there is little fencing at Langholm and collisions account for fewer than 1% of all recorded deaths³. Second, Highland studies⁵ suggest that 11% of red grouse deaths are due to collisions but 48% are due to raptor predation. Third, fences pose fewer problems to red grouse than to woodland grouse as strikes are

concentrated near young plantations⁴ and red grouse are birds of open moorland.

Finally, how can conflicts between raptors and grouse shooting be resolved¹? As suggested by Mead⁶, predation patterns observed at Langholm will not apply everywhere. Our data suggest that, in the absence of persecution, harrier numbers will be related to densities of prey other than grouse³. In the long term, reducing the amount of grassland on moors may reduce the numbers of songbirds and voles, leading to reductions in harrier density and reducing their impact on grouse populations.

In the short term, however, raptor–grouse problems may require more active intervention in the form of supplementary feeding or raptor translocation. Such measures require the cooperation of conservation and shooting interests. The future of raptors, grouse, the moorland habitats they share and the rural communities they support depends upon it.

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1. May, R. M. *Nature* **389**, 330–331 (1997).

2. Mead, C. *Nature* **389**, 780 (1997).

3. Redpath, S. M. & Thirgood, S. J. *Birds of Prey and Red Grouse* (HMSO, 1997).

4. Baines, D. & Summers, R. W. J. *Appl. Ecol.* **34**, 941–948 (1997).

5. Hudson, P. J. *Grouse in Space and Time* (Game Conservancy Trust, 1992).

6. Mead, C. *The Times*, 7 & 23 November 1997.

Exaggeration or underestimate?

Sir — John Maddox urged caution in approaching the Kyoto conference on climate change for three reasons (*Nature* **390**, 111; 1997). In each of these reasons he is seriously mistaken.

(1) He suggests that the predictions of the Intergovernmental Panel on Climate Change (IPCC) may exaggerate the rate of change by a factor of two. The fact is that they may underestimate the rate of change by a factor of two. The potential for an underestimation seems especially great because of the uncertainties of biotic responses, especially the possibility of releasing large quantities of additional carbon as carbon dioxide and methane

from high-latitude forests and tundra.

(2) He suggests that the IPCC has not provided a study of the effects on the global economy of restricting the use of fossil fuels. He is correct, but there are ample studies to show that the transition can be made with great advantage. I suggest he consults a recent study by the World Resources Institute led by Dr Robert Repetto.

(3) He suggests that the problem of inequity between rich and poor countries has not been resolved. The issue will never be resolved to the satisfaction of all, but there is good basis for believing that the less-developed world can leap over the fossil-fuel age into an era of far more efficient use of energy with reliance on enduring sources. The developed world can and should aid this transition. The gains will

be mutual. Again, there will always be an argument on the basis of equity, but we have never previously allowed such arguments to prevent major transitions in human affairs.

The Kyoto meeting has been dealing with the most important social and political issue of our time. It is essential that systematic and rapid progress be made towards stabilizing, not the emissions, but the atmospheric burden of heat-trapping gases. The cost of failure is progressive environmental impoverishment and political chaos.

George M. Woodwell

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A little learning

Sir — In his evaluation of Steven Rose's book *Lifelines: Biology, Freedom, Determination* (*Nature* **390**, 36; 1997), Benno Müller-Hill argues strongly against Rose's thesis that excessive reductionism is a serious problem in today's biology. He then goes on to say: "In fruitflies, the first stages of development are now well understood and in fish we may know them soon."

This is a ridiculous statement in view of our ignorance of so many basic principles of cells and their interaction. It is presumably the kind of attitude that Rose is critical of: the notion that if you know the genes, you know everything. In my view, it represents an extreme example of *reductio ad absurdum*.

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Open access to data

Sir — The European Database Directive EU 96/9 comes into force in January 1998. It gives copyright protection for electronic and other databases, but brings serious

implications for science. Its philosophy confronts the widely accepted principle of 'full and open access to data' at minimum cost, which is vital to sciences such as astronomy, solar-terrestrial and space physics, and geophysical and environmental sciences.

To protect science, I suggest that 'full and open access to scientific data' should become a declared national policy, to which exceptions, for commercial or other reasons, have to be justified.

Many research councils and other agencies do place their scientific data in the public domain, as indeed is required for some categories of data by, for example, the Antarctic Treaty, the European Space Agency convention, and European Commission directive 90/313 on environmental data. The new legislation, however, raises concerns.

First, discussions in US circles suggest that the new all-embracing '*sui generis*' ('database right') may enable 'rights' to data to be hijacked by contractors or other parties involved in the data-handling chain. It therefore behoves research organizations to make sure that their past and future data are secure against any such possibility.

Second, the applied research that often follows from 'academic' research could be seriously hampered unless any required data are in the public domain, as the

European Union allows no 'fair use' exceptions to database copyright for research regarded as 'commercial'.

Furthermore, *all* 'fair use' (or 'fair dealing') exceptions from copyright, even for the purposes of teaching or research, are at grave risk. Commercial interests are pressing strongly for *no* 'fair use exceptions' whatever in the United Kingdom.

The Patent Office, which is responsible for the UK legislation to implement EU 96/9, has been inquiring into the economic cost and benefits of such a policy. The terms of this inquiry load the scales against 'fair use', because it is almost impossible to quantify the negative effects of forcing teachers and scientists to buy licences for each and every use of copyright material (apart from a nebulous right to extract 'insubstantial parts' of a database). A strong case is needed to save 'fair use'.

Incidentally, the wide-ranging definition of 'database' includes printed matter, so even the use of a book may fall foul of '*sui generis*'.

Henry Rishbeth

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An early warning of the risks of rocketry

Sir — The recent study by M. N. Ross *et al.* on possible stratospheric pollution by rockets (*Nature* **390**, 62–64; 1997) indicates purely local destruction of the ozone layer, with little impact on the overall geographical picture.

Probably the first engineer to alert the international community to the danger of atmospheric pollution by rockets fired into the upper atmosphere was an Englishman, C. F. Dendy Marshall, who, in a letter of 16 February 1932 to the League of Nations, called for international intervention. He was responding to an article in *The Times* of London of 25 August 1931 that referred to the intention of Dr Darwin O. Lyon to fire a rocket to a height of 50 miles for scientific purposes.

Marshall drew attention to the existence of hydrogen at remote altitudes which without oxygen would not burn, but he warned of the probability of “an explosive belt which the rocket would set on fire in its passage”.

He went on to say: “The effect might merely be an unprecedented display of the nature of an aurora. On the other hand, the heat generated might be so great as to bring

about the end of the world, so far as we are concerned. There is a risk of wiping everybody out, except those of us who were in the Tube, together with such miners as were at work....”

Marshall quoted Sir James Jeans’s estimates that the proportion of hydrogen to oxygen molecules in a given volume of air at ground level was 1:21,000; at a height of 12 miles, 1:875; and at 50 miles about 17:1.

Assuming that the mixture would be flammable between 25 and 30 miles, Marshall calculated that the affected volume would be 47,666 cubic miles, albeit at a very low mean pressure of 0.035 pounds per square inch. The equivalent at ground level pressure would be 113 cubic miles, “enough for a good explosion”.

Paul Tunbridge

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Nuclear agency’s research misjudged

Sir — In your News story “Nuclear agency may absorb physics institute” (*Nature* **389**, 895; 1997), you say “the move is intended to increase CEA’s [France’s atomic energy

commission’s] fundamental research capacity, which is widely considered to lag behind the commission’s engineering prowess”.

Fundamental research is one of the important and recognized goals of CEA, and is carried out in all its divisions, and in particular in the Direction des Sciences de la Matière (‘matter’, not ‘materials’), devoted to basic research in physics and chemistry.

Our institute employs 1,800 CEA staff, and there are 1,200 permanent employees in our laboratories from other agencies or universities, visitors and graduate students. The departments are evaluated every two years by international committees chaired by distinguished scientists.

Together with excellent advice, our laboratories have received much praise from these committees, and many DSM scientists have received national and international awards.

I must also say that I was somewhat surprised by your harsh report, given that our results are often published in *Nature*.

Catherine Cesarsky
(Director)

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How to evaluate journal impact factors

Sir — Journal impact factors, published annually by the Institute of Scientific Information (ISI) in two *Journal Citation Reports* (JCR) editions (science and social science), are a well known — though not uncontested^{1,2} — means of evaluating and comparing the scientific impact of journals. A major disadvantage of the JCR is the limited number of evaluated journals. For example, the 1996 JCR on CD-ROM (science edition) lists 4,779 journals, roughly half of them with biomedical orientation.

In contrast, about 4,000 journals are continuously indexed in Medline, nearly 3,500 in Embase and at least 4,600 in Biosis, making a total (without duplicate titles) of about 7,000 unique journals in these three biomedical databases (data retrieved from Serline).

There is, however, an easy way to construct impact factors also for journals not included in the JCR. Online database searches can be carried out to determine the number of published articles in a given journal and the number of citations to that journal, according to ISI's definition of a journal's impact factor (number of citations in year X to papers published in the journal in question in years X-1 and X-2, divided by the number of counted articles).

The table shows constructed impact factors (CIF) for several biomedical journals not included in the JCR 1996 science edition (No. 1-11). In addition, to confirm the reliability of the method, the constructed as well as the JCR-derived impact factors (JCR-IF) are shown for a couple of journals included by JCR (No. 12-17). All online searching was carried out at the German host DIMDI (Deutsches

Institut für Medizinische Dokumentation und Information). Numbers of articles published in 1994 and 1995 were retrieved by searching the databases Medline, Cancerlit, Healthstar, Embase, Biosis and Scisearch simultaneously (DIMDI's 'superbase' modus), followed by elimination of duplicate articles using the 'check duplicates' command of DIMDI's retrieval language. Editorials, letters, news and meeting reports were excluded from searches because they are not subject to ISI's citation analyses³. Numbers of citations were retrieved from ISI's database Scisearch which — in addition to bibliographic data — contains all the references cited in the indexed articles, whether the cited journal is being indexed in Scisearch or not.

The slight differences between constructed and JCR-derived impact factors may be due to the following: (i) for retrieval of published articles the document types 'article', 'journal article' and 'review' were used, which may have a wider definition in non-ISI databases, thus leading to higher article counts (and lower CIFs) than the more restrictive use of the term 'research article' seen in JCR 4; and (ii) Scisearch comprises more journals than JCR (personal communication from ISI) which may lead to higher citations.

Impact factors can only give some hints with respect to the importance of a journal in its field. Nevertheless, because there is permanent pressure on scientists to prove the impact of their work and to publish in journals with measurable impact factors, the method described might also be helpful in evaluating those journals not listed in JCR.

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Constructed impact factors for journals not in JCR

No.	Journal	Articles published 1994+1995	Citations 1996	CIF	JCR-IF
1	Advances in Neurology	122	74	0.607	-
2	Advances in Pharmacology	180	434	2.411	-
3	American Journal of Dentistry	143	203	1.420	-
4	Annals of Clinical Psychiatry	68	47	0.691	-
5	Brain Topography	59	55	0.932	-
6	British Journal of Clinical Psychology	111	35	0.315	-
7	Current Topics in Pathology	35	27	0.771	-
8	International Dental Journal	124	95	0.766	-
9	International Journal of Health Services	89	21	0.236	-
10	Morbidity and Mortality Weekly Report	774	147	0.190	-
11	Stereotactic and Functional Neurosurgery	171	62	0.363	-
12	Abdominal Imaging	287	202	0.704	0.733
13	Cellular Immunology	573	1,243	2.169	2.142
14	International Journal of Developmental Biology	201	352	1.751	1.702
15	Molecular Medicine	81	304	3.753	3.972
16	Nature	1,994	54,024	27.093	28.417
17	Transplant International	165	238	1.442	1.522

1. Moed, H. F & van Leeuwen, Th. N. *Nature* **381**, 186 (1996).
2. Opthof, T. *Cardiovasc. Res.* **33**, 1-7 (1997).
3. *Journal Citation Reports* (Science ed.) 1996 on CD-ROM. Citation and Article Counts.
4. *Journal Citation Reports* (Science ed.) 1996 on CD-ROM. Using the JCR Wisely.

Standards for safety cabinets

Sir — In the past few years, protection from potential airborne hazards associated with handling microbiological agents in the laboratory has owed much to the improvement in containment performance of microbiological safety cabinet installations after the smallpox outbreak in the United Kingdom in 1978.

A new European safety cabinet standard is now being proposed to cover the whole field of biotechnology. Early in the discussions for the standard, it was agreed that UK standard BS 5726 (1992) would form the basis of the European Union (EU) standard.

It has recently been suggested, however, that type testing and the measurement of safety cabinet airflows, together with some site evaluation (but no measured validation) is sufficient to guarantee worker protection without the need for on-site containment tests.

In my opinion, this will be a retrograde and dangerous step. A recent survey of test results over 12 months from one major independent UK test house showed that 37 class II safety cabinets (all with adequate type test certification and including 18 new installations) failed to meet the BS 5726 operator protection factor requirements.

In all cases where containment failed, problems of the cabinet or the environment were identified and remedial action taken. Without on-site containment tests, potentially dangerous equipment would have been in service.

The new performance standard for microbiological safety cabinets is being developed under the banner of biotechnology, but it is likely that its use will extend to all other areas where microbiological material is being handled, particularly when related national standards in EU countries are withdrawn under the EU standardization rules.

It is vital that, in the context of the proposed safety cabinet standard, testing for containment both at commissioning and during routine maintenance remains a fundamental requirement.

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The fastest way from A to B

Tony Sudbery

Quantum teleportation is a way of transferring the state of one particle to a second, effectively teleporting the initial particle. This technique has only existed as a thought experiment – until now.

Quantum teleportation has been achieved in the laboratory, by teams headed by Anton Zeilinger in Vienna (who report on page 575 of this issue¹) and Francesco De Martini in Rome² (in a paper submitted to *Physical Review Letters*). This fancifully named process, devised four years ago by Charles Bennett *et al.*^{3,4}, is a way to transfer quantum information about an object instantaneously to a distant object, using 'entanglement' — a mysterious connection between separated objects, which Einstein, Podolsky and Rosen pointed out was a feature of quantum mechanics. Einstein *et al.* found entanglement unbelievable, but thanks to John Bell and the experiments inspired by his work, it is now well established. It is also accepted that entanglement cannot be used to transfer readable information instantaneously, which would violate the principles of relativity. Indeed, what Bennett *et al.* showed is that the full information needed to reconstruct the state of an object can be divided into two parts, quantum and classical. The former can be transmitted instantaneously, but it cannot be used without the latter, which can only be transmitted by conventional means at the speed of light or slower.

The communication channel for teleportation consists of a pair of entangled particles, one held by the sender, Alice, and one by the receiver, Bob. Let us call these 'handset' particles. The entanglement is like an invisible cat's cradle connecting them, and it is delicate — the particles must be well isolated from their environments to remain in a coherent entangled state. A third party, Carol, gives Alice another particle whose state, constituting the message, is to be communicated to Bob. Alice cannot simply read the message and transmit the information by a conventional channel, because quantum mechanics decrees that the full information is not accessible on a single reading, and the process of reading will change some of the information unpredictably. Instead she measures a joint property of the message particle and her handset. This still leaves her with incomplete information about the message and causes an uncontrollable change in the particles. But because of the entanglement, it simultaneously causes a related change in Bob's handset particle. This is the

quantum part of the information. The classical part is the result of Alice's measurement, which she must communicate by a classical channel. It tells Bob what operation he must perform on his handset to complete the transformation and make it into a perfect copy of the message.

The practical difficulty lies in measuring the joint property of the message particle and Alice's handset particle. How can the two be

held together during the measurement? The two experiments find different ways around this. In Zeilinger and colleagues' experiment (Fig. 1), Alice's handset particle and Carol's message particle are directed at the joint measurement apparatus independently, with no guarantee that they will arrive together. Only in the rare cases that they coincide is the joint measurement made and the teleportation successful. De Martini's experiment (Fig. 2) has a much higher proportion of successes, but demonstrates a slightly different kind of teleportation, suggested by Sandu Popescu². In this, the message particle and Alice's handset, instead of being two separate particles, are two aspects of a single particle, namely the polarization and direction of motion of a photon. These enter in the theory just like two separate particles, and they can be used just as well to demonstrate teleportation. The only difference is that Carol must inscribe her message on Alice's handset instead of giving it to her on a separate particle. ▶

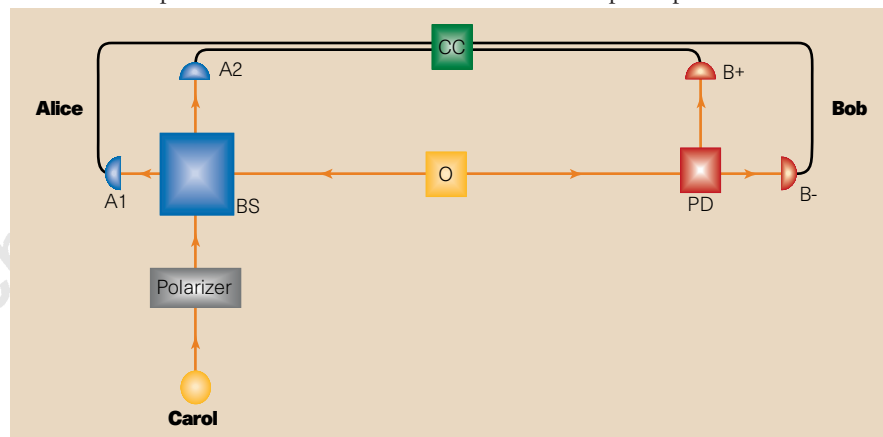


Figure 1 The teleportation experiment of Zeilinger and colleagues¹. O is the source of an entangled pair of photons; BS is Alice's beamsplitter with detectors A1 and A2 at its output ports; PD is Bob's polarization detector (with output B+ corresponding to the polarization of Carol's message particle). A coincidence counter (CC) registers triple coincidences between A1, A2 and B+, but not A1, A2 and B-. Carol's message particle has effectively been teleported to Bob's polarization detector.

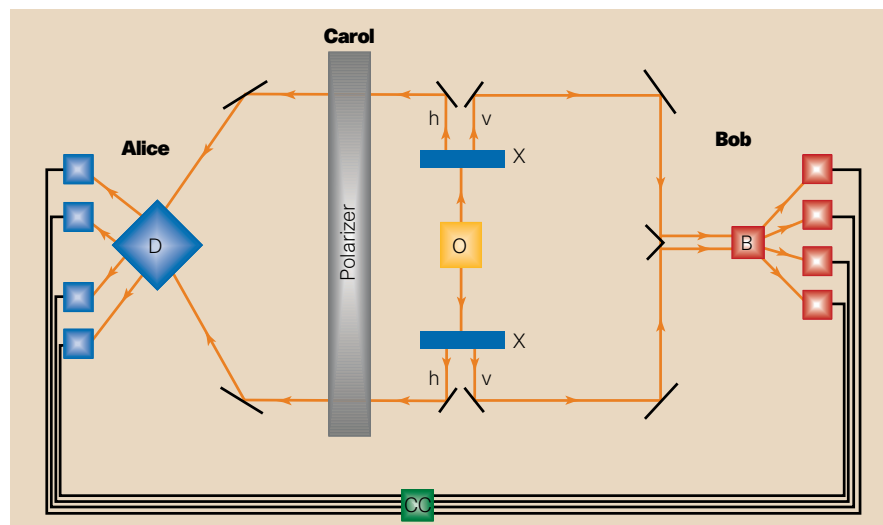


Figure 2 The experiment of De Martini and colleagues². Calcite crystals (X) separate horizontally and vertically polarized photons. Bob's switch (B) sends his photon to four detectors, each corresponding to one outcome of Alice's detector (D); and the counter (CC) detects coincidences between the two.

In the experiment of Zeilinger and colleagues, pairs of entangled photons are created by a process called parametric down-conversion, in which a single photon is converted into two plane-polarized photons with perpendicular planes of polarization. These are pairs of handset particles, so one is sent to Alice, the other to Bob. Independently, a photon of the same frequency is put in a known state of polarization and sent to Alice to be the message particle. Alice has a beamsplitter, a device that can receive light in two entry ports and direct it to either of two exit ports. Her joint measurement on the two particles she receives consists of directing them to the two entrance ports of her beamsplitter and seeing how they emerge. This has the desired effect on the particles (and therefore on Bob's handset particle) if they arrive simultaneously. The particles were detected by Zeilinger and colleagues if they emerged from different output ports of the beamsplitter with opposite polarizations, which is one of the four possible outcomes. In those cases, Bob's handset particle was found to acquire the state of the message particle, and the particle had thus been teleported.

De Martini's group proceeds differently. They first convert the correlation between the polarizations of the entangled pair to a correlation between their paths in space. Each handset particle can travel by two different routes to its recipient Alice or Bob, and if one takes the high road, the other takes the low road. This frees the polarization aspect of Alice's photon to be the message. On its way, it is put in a chosen state of either plane or elliptical polarization — this is Carol

inscribing her message. At Alice's end, both routes lead the photon to a device that measures various combinations of the polarization of the photon and its path of travel. Meanwhile, Bob's photon goes to a detector with four possible settings, each corresponding to one result of Alice's measurement: for a particular setting, the detector fires if the operation called for by the corresponding result of Alice's measurement would put the photon in the polarization state inscribed by Carol. Thus if teleportation has been successful, Bob's detector will fire every time the corresponding result is obtained by Alice — as was observed.

Quantum teleportation is a striking application of the holistic nature of the physical world revealed by quantum mechanics. It is expected to have uses in quantum computers, which will, in theory, be much faster than classical computers at solving certain problems. It can be used to transmit information reliably in noisy situations where messages would otherwise be degraded, and to transfer information from fleeting or hard-to-control carriers, such as photons, to particles more suitable for permanent storage, such as trapped ions. And it is bound to feature in the continuing discussions about realism, locality and whether there really is what Einstein called "spooky action at a distance". □

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1. Bouwmeester, D. *et al.* *Nature* **390**, 575–579 (1997).
2. Boschi, D., Branca, S., De Martini, E., Hardy, L. & Popescu, S. *Phys. Rev. Lett.* (submitted).
3. Bennett, C. H. *et al.* *Phys. Rev. Lett.* **70**, 1895–1899 (1993).
4. Sudbery, T. *Nature* **362**, 586–587 (1993).

Learning and memory

Never fear, LTP is hear

Robert C. Malenka and Roger A. Nicoll

Synaptic transmission is the main mechanism by which neurons communicate, and an attractive hypothesis is that learning occurs, and memories are encoded, by activity-dependent changes in synaptic strength. With the discovery of hippocampal long-term potentiation (LTP), and the ability to study its mechanisms in experimentally accessible *in vitro* preparations, neurobiologists seemed to be on the verge of solving one of the more fascinating problems in neurobiology — the molecular basis for memory. But some reports have raised a nagging doubt as to whether LTP-like phenomena are actually used in the behaving animal during learning¹. Now, complementary papers by Rogan *et al.*² and McKernan and Shinnick-Gallagher³ (pages 604 and 607 of this issue) lay this doubt to rest. They show that fear conditioning, which is a form of pavlovian (classical) con-

ditioning, indeed causes an increase in synaptic strength in the appropriate neural circuit.

Why has it been so difficult to make a connection between LTP and memory? Most studies have concentrated on the hippocampus, because this medial temporal lobe structure was the first one shown to be part of the brain's memory system⁴. Moreover, LTP was first described in the hippocampus⁵, and it is most easily studied in this structure. However, the exact functions of the hippocampus remain unclear and the hippocampal-dependent tasks that are often used in behavioural studies are complex. Thus, the exact information carried by the hippocampal synapses and circuits that are (presumably) modified during learning and memory is unknown. Combining sophisticated molecular genetic and electrophysiological techniques in the behaving animal

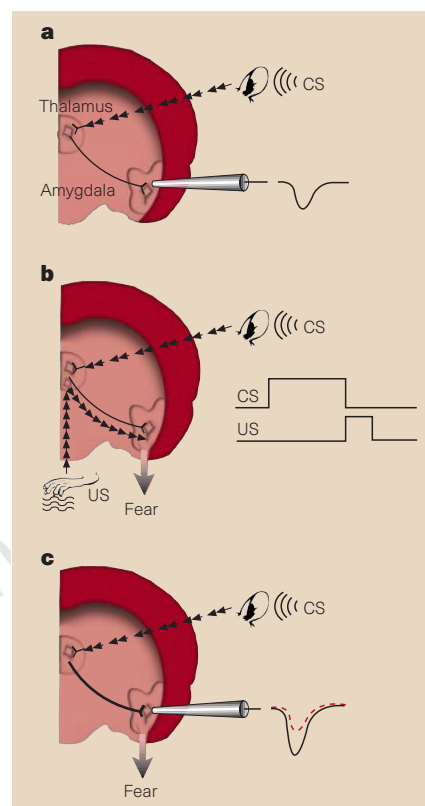


Figure 1 Experiment by Rogan *et al.*² to show the link between long-term potentiation and memory, using a fear-conditioning response. The authors monitored changes in the extracellular potential in the lateral amygdala of an animal while it was trained to respond to a conditioned stimulus (CS), which was an audible tone, and/or an unconditioned stimulus (US; a foot shock). a, Pre-training. The sound waves (the CS) go into the ear, and this information goes to the thalamus and then to the amygdala, where a response is recorded. b, During training. The US is given just after the CS, and this causes 'fear'. c, After training. The CS now causes a larger response in the amygdala, and alone also elicits fear. The pathway from the thalamus to the amygdala is thicker, because this is the path that has presumably been strengthened during the training. (Dashed lines represent neural pathways that are either not illustrated or not known.) In a complementary study, McKernan and Shinnick-Gallagher³ have shown *in vitro* that fear conditioning causes an increase in strength at the synapses that process the CS in the lateral amygdala.

has been a step in the right direction⁶. But the demonstration of an increase in hippocampal synaptic strength (that is, LTP) during learning has remained elusive.

Enter fear conditioning, which has many attractive features for studying the neural mechanisms of memory. During fear conditioning, a neutral conditioned stimulus (the CS; for example, a tone) is followed by an unconditioned stimulus (the US; for example, a foot shock) which routinely elicits a

stereotypic response — the behavioural correlates of fear. After one or several pairings, a robust memory of the CS-US relationship is formed. So the CS alone can elicit the full behavioural repertoire that was previously produced only by the US. Conditioning is thought to require changes in the pathway that mediates the CS, due to the convergence of inputs from the US (ref. 7).

The lateral amygdala (LA) is thought to be the critical structure in which information from the CS and US converge (Fig. 1)⁸. Because the CS is a simple sensory stimulus, the afferents that carry the CS information into the LA can be defined. This connection between the auditory thalamus and LA can express LTP (ref. 9) which, when induced with electrical stimulation, causes an increase in the response of the LA to auditory stimulation¹⁰. The processing of natural stimuli can, therefore, use the mechanisms set up by artificially induced LTP.

But does LTP occur at thalamo-LA synapses during fear conditioning? To address this, Rogan *et al.*² monitored the extracellular potential in the LA, in response to the CS tones while a rat was trained. As the CS and US were paired, and the animal learned to respond to the CS with a behavioural correlate of fear, the response in the LA to the CS alone grew, and remained at a high level. Further presentations of the CS alone extinguished the behavioural response (that is, the memory), and the auditory-evoked potential returned to baseline. Importantly, when the CS and US were unpaired — so no learning occurred — there was no significant growth in the auditory-evoked potential.

These data compellingly show that there was a change in the pathway which processes the CS as the animals learned to associate the CS and US. Enhancement of the auditory-evoked LA response was identical to that elicited by electrical induction of LTP in the thalamo-LA path¹⁰. So the authors propose that the critical change occurred at the synapses in the LA. However, changes in other auditory processing stations, or in the intrinsic electrical properties of LA neurons, may have caused the behaviourally induced changes. Furthermore, the increase in the auditory-evoked response was not shown to be specific for the CS, as opposed to reflecting some general increase in LA responsiveness.

By preparing *in vitro* slices of the LA from fear-conditioned rats, McKernan and Shinnick-Gallagher³ examined the synaptic responses of LA neurons to stimulation of afferents from the auditory thalamus. These responses were consistently larger than those recorded from LA slices prepared from control animals that had undergone unpaired CS-US training. Furthermore, the synaptic responses of LA neurons to an independent input that was not involved in fear conditioning remained unaltered. The compelling

conclusion is that fear conditioning caused an increase in synaptic efficacy (for example, LTP), specifically at the synapses that process the CS. The authors also suggest that this behaviourally induced increase in synaptic strength may be due, at least in part, to presynaptic modifications, because it was accompanied by a change in one form of short-term presynaptic plasticity.

Is the LTP-memory connection — at least for one form of learning — now established enough to silence the sceptics? Rogan *et al.*² point out several features common to fear conditioning and hippocampal LTP, including their dependence on NMDA (N-methyl-D-aspartate) receptors^{11,12}. Nevertheless, it remains to be shown that the mechanisms responsible for the behaviourally induced synaptic changes are the same as those underlying electrically induced LTP. But the new reports^{2,3} indicate that attempts to study LTP have not simply been an intellectual exercise, and that

progress continues towards a comprehensive understanding of the mechanisms that underlie learning and memory. □

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1. Eichenbaum, H. *Nature* **378**, 131–132 (1995).
2. Rogan, M. T., Stäubli, U. V. & LeDoux, J. E. *Nature* **390**, 604–607 (1997).
3. McKernan, M. G. & Shinnick-Gallagher, P. *Nature* **390**, 607–611 (1997).
4. Scoville, W. B. & Milner, B. *J. Neurol. Neurosurg. Psychiatr.* **20**, 11–21 (1957).
5. Bliss, T. V. P. & Lomo, T. *J. Physiol. (Lond.)* **232**, 331–356 (1973).
6. Morris, R. G. M. & Morris, R. J. *Nature* **385**, 680–681 (1997).
7. Pavlov, I. P. *Conditioned Reflexes* (Dover, New York, 1927).
8. LeDoux, J. E. *Annu. Rev. Psychol.* **46**, 209–235 (1995).
9. Clugnet, M.-C. & LeDoux, J. E. *J. Neurosci.* **10**, 2818–2824 (1990).
10. Rogan, M. T. & LeDoux, J. E. *Neuron* **15**, 127–136 (1995).
11. Miserendino, M. J. D., Sananes, C. B., Melia, K. R. & Davis, M. *Nature* **345**, 716–718 (1990).
12. Gewirtz, J. C. & Davis, M. *Nature* **388**, 471–474 (1997).

Genome sequencing

New tricks of tick-borne pathogen

Alan G. Barbour and Wolfram R. Zückert

Lyme disease is the most common vector-borne disease in Europe, the United States and parts of Asia^{1,2}. In North America, the infection is transmitted by deer ticks between small mammals or, inadvertently, to people (Fig. 1). Like HIV and other emerging infections of the late twentieth century, the aetiological agent — *Borrelia burgdorferi* — was unknown to the last generation of microbiologists and physicians. Now, before we are even close to understanding the pathogenesis of Lyme disease, Fraser *et al.*³ report the near-complete sequence of the *B. burgdorferi* genome on page 580 of this issue. These findings are a first in several respects: this is the first genome from the eubacterial phylum of spirochaetes, with their peculiar morphology, physiology and behaviour; it is

the first genome of a parasite that infects both invertebrates and vertebrates; and it is the first genome of a prokaryote with several genetic elements, most of which are linear.

But those who were expecting to find in *B. burgdorferi* a rich vein of gold in which to mine virulence determinants have to be disappointed. The sequence is as notable for what it does not contain as for what it does. Instead of finding many orthologues⁴ of toxin and invasion genes, global regulatory systems, two-component signal-transduction pathways and bacteriophages of other pathogenic bacteria, the authors found an almost bewildering array of duplicated lipoprotein genes, unique to *Borrelia* spp. and of unknown function. These genes are located on extrachromosomal stretches of

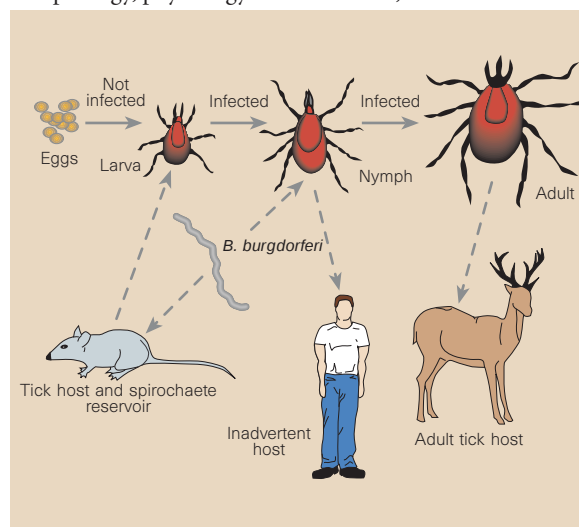


Figure 1 Life cycle of *Ixodes* tick vectors of *Borrelia burgdorferi*, the spirochaete agent of Lyme disease that has now been sequenced by Fraser *et al.*³. Small rodents, such as mice, are reservoirs for *B. burgdorferi*. The tick becomes infected from feeding on a mouse and remains infected as it changes to nymph and then adult. The spirochaetes are transmitted by infected nymphs to other mice and to humans, which are inadvertent hosts. Deer are important hosts for adult ticks, but are not effective reservoirs for *B. burgdorferi*.

DNA called plasmids. One of the lipoproteins, OspA, has already been crystallized and structurally characterized⁵, and it is undergoing human field trials as a vaccine against Lyme disease, although no one yet knows what it does.

Discovery of the linear chromosome in *Borrelia*^{6,7} challenged ideas of what a bacterial chromosome is. The findings of Fraser *et al.*³ now provide further evidence that the distinction between a plasmid and chromosome is primarily a matter of size. The linear and circular plasmids of *Borrelia* spp. are equimolar with chromosomes^{8,9}, they contain genes that are usually found on chromosomes (for example, *guaA* and *tRNA^{Gly}*), and they have apparently undergone recombination with the chromosome. These elements could equally be examples of minichromosomes as of plasmids, and they seem to provide an opportunity and place for the organism to duplicate genes and rearrange them without much cost or damage. Moreover, by possessing several copies of highly similar genes on the same (and different) elements, there is the potential for diverse antigenic variation — and this has been observed in relapsing fever *Borrelia* spp.¹⁰.

Determination of the *B. burgdorferi* genome has opened doors for investigation and closed just as many. By understanding the biosynthetic and transport limitations of *B. burgdorferi*, we may be able to develop a medium in which to grow as-yet uncult-

ivable *Borrelia* spp. The results encourage study of a more metabolically competent spirochaete, such as the free-living *Spirochaeta aurantia*, for a better understanding of how this ancient group of bacteria evolved, and to identify catalytic molecules of industrial importance. But the sequence does not explain the persistence of the disease in some people yet not in others; the differential expression of surface proteins in the tick and in the mammal; or migration of the spirochaetes from the midgut to the salivary gland of the tick, or from the skin to the brain of the mammal. These explanations will require taking the hints (and primers) from the genome and returning to animal models and the clinics. □

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1. Barbour, A. G. & Fish, D. *Science* **260**, 1610–1616 (1993).
2. Nocton, J. J. & Steere, A. C. *Adv. Intern. Med.* **40**, 69–117 (1995).
3. Fraser, C. M. *et al. Nature* **390**, 580–586 (1997).
4. Henikoff, S. *et al. Science* **278**, 609–614 (1997).
5. Li, H., Dunn, J. J., Luft, B. J. & Lawson, C. L. *Proc. Natl Acad. Sci. USA* **94**, 3584–3589 (1997).
6. Ferdows, M. S. & Barbour, A. G. *Proc. Natl Acad. Sci. USA* **86**, 5969–5973 (1989).
7. Baril, C., Richaud, C., Baranton, G. & Saint Girons, I. *Res. Microbiol.* **140**, 507–516 (1989).
8. Hinnebusch, J. & Barbour, A. G. *J. Bacteriol.* **174**, 5251–5257 (1992).
9. Casjens, S. & Huang, W. M. *Mol. Microbiol.* **8**, 967–980 (1993).
10. Restrepo, B. I. & Barbour, A. G. *Cell* **78**, 867–876 (1994).

Stellar astronomy

Clocks within the hourglass

Mario Livio

Rarely in astronomy can a stellar cataclysm be predicted in advance. It is also rare that an extremely well-studied object suddenly reveals a previously undetected periodicity. But both of these things are true for one of the most famous stars in the sky, the luminous blue variable Eta Carinae. On page 587 of this issue¹, Corcoran *et al.* report the discovery of small-scale X-ray outbursts from this source, which repeat every 85 days, superimposed on an overall increase in the mean X-ray flux, which may herald a dramatic 'event' in January.

Luminous blue variables (LBVs) represent a short-lived phase in the evolution of massive stars, during which the star occasionally erupts, changing markedly in its brightness and spectrum. The variations typically fall into three categories: 'micro' variations, up to a few tenths of a magnitude (one magnitude is a factor of about 2.5) on timescales of weeks to months; minor eruptions (increases in brightness by about one magnitude on timescales of years); and giant outbursts (increases by more than three magnitudes, with durations of decades²).

LBVs may be an evolutionary stage between massive 'O-type' stars that are still burning hydrogen in their cores, and Wolf-Rayet stars, which have run out of hydrogen and must burn helium instead.

Eta Carinae is the most famous and the most spectacular LBV. But despite many observations, covering almost all wave bands, its exact nature remains a mystery. In particular, no satisfactory explanation exists for its huge outburst in the first half of the last century. (Some of the more promising mechanisms involve the action of strong radiation pressure on layers with high opacity.) That eruption resulted in the ejection of an hourglass-shaped bipolar nebula, now called the Homunculus³ (Fig. 1, overleaf).

A remarkable recent discovery about Eta Carinae is a probable 5.5-year periodicity in its spectrum^{4,5}. The discovery of a periodicity often leads to a detailed dynamical model of a system; and in the case of Eta Carinae, the 5.5-year period could either be the orbital period of a highly eccentric binary, or a timescale associated with the stability of a single star^{6,7}. The local thermal timescale of some part of



100 YEARS AGO

Astronomy is, perhaps, among all physical sciences the one destined by its historical tradition, no less than by its present and future necessities, to second – nay, to promote and develop – the cosmopolitan tendency. The grand spectacle of the face of the heavens, ever before the eyes of all; the difference of phenomena according to the horizons, which carries with it the need of co-operation between the observers diversely situated with regard to the celestial sphere; in fine, the high and significant moral education that comes to astronomers from the continual contrast between the immensity of the heavens and the miserable narrowness of the limits traced out conventionally on the globe between one country and another; here are the causes through which a spirit superior to any narrow nationalism was soon breathed into our souls. Tycho Brahe, the proud Danish patrician, the founder of practical astronomy in the Renaissance, sings sternly –

Omne solum forti patria est, coelumque Undique supra....

And his name, with those of Copernicus, Kepler, Galileo and Newton, form a constellation that shines not more for the sky of Denmark, than for that of Germany, Italy, or England!

From *Nature* 9 December 1897.

50 YEARS AGO

"Coconut Conference" – Because of the present world shortage of vegetable oils and fats, and the fact that the situation is one which will take years to remedy, particular interest is attached to oil-producing crops, their cultivation, conservation, potentialities and use. During this summer the Coconut Conference (Colombo, July 4, 1947) covered much useful ground, papers, chiefly of a directly practical nature, being devoted to the regeneration of plantations, the use of manures, the value of combining animal husbandry with coconut cultivation, problems of soil conservation, the control of insect pests, and problems of co-operation and marketing of coconut products. This report will undoubtedly prove of interest and value to scientific workers and members of the planting community in other tropical regions where the coconut is an important crop.

From *Nature* 13 December 1947.

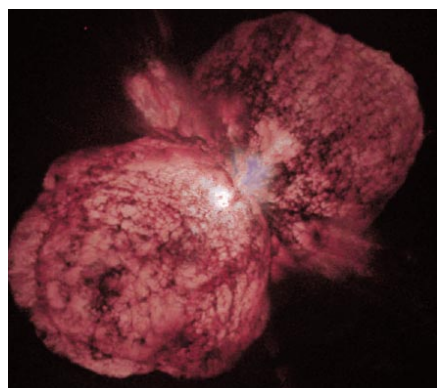


Figure 1 Eta Carinae, a century and a half after its last giant eruption. This exceptionally massive star — perhaps a hundred times the mass of the Sun — is only marginally stable. It underwent a massive outburst in the early part of the nineteenth century, becoming one of the brightest stars in the sky, despite being more than 8,000 light years away, and ejecting these two huge lobes of gas and dust. A new ‘event’, probably not as dramatic as the last one, is likely to happen in January.

the star, for example, could be the time between shell ejections in a dense wind.

If Eta Carinae is a binary, then its X-ray emission may be produced by the collision of the two stellar winds. This collision will cause shock waves that heat the gas to temperatures high enough to emit X-rays. But to produce X-rays in the 2–10-keV band, the winds must have speeds in excess of 2,000 km s⁻¹, which is slightly higher than would be expected from these stars. More importantly, if it is indeed an eccentric binary, then the observed increase in X-ray emission (which has accelerated since January) may signify an imminent closest approach, at which point the emission is expected to reach a maximum. Even if the 5.5-year period is merely from the variation of a

single star, a dramatic ‘event’ (in terms of X-ray luminosity and the spectral behaviour) is still expected to occur this coming January.

The 85-day period is equally enigmatic. It could be, among other things, the orbital period of a close binary, the rotational period of a single star, or a stellar pulsation period. But given the simultaneous 5.5-year period and the size of the star (70–100 solar masses), all of these potential explanations have problems. For example, 85 days is much longer than a typical pulsation period of such a star (though the fact that Eta Carinae is only marginally stable makes this uncertain). And if 85 days is simply the stellar rotation period, then it is not clear how the X-ray ‘mini-outbursts’ are produced.

As we know when the ‘big event’ is expected to occur, there is an unprecedented opportunity to coordinate a multi-wavelength monitoring campaign. That could prove or disprove the binary nature of Eta Carinae. In the binary model, the X-ray flux should continue to increase towards closest approach, and then rapidly decline. Also, the ultraviolet flux should decrease during the event, and then increase again, as the secondary star passes behind the primary and the wind from the latter blocks most of the secondary’s UV. We may finally be near to solving the mystery of Eta Carinae. □

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1. Corcoran, M. F. *et al.* *Nature* **390**, 587–589 (1997).
2. Bohannan, B. in *Luminous Blue Variables: Massive Stars in Transition* (eds Nota, A. & Lamers, H. J. G. L. M.) 3–7 (Astron. Soc. Pacif., San Francisco, 1997).
3. Morse, J. *et al.* STScI press release STScI-PRC96-23 (1996).
4. Damiani, A. *Astrophys. J.* **460**, L49–L52 (1996).
5. Damiani, A., Conti, P. S. & Lopes, D. F. *New Astron.* **2**, 107–117 (1997).
6. Davidson, K. *New Astron.* **2**, 387–390 (1997).
7. Davidson, K. & Humphreys, R. M. *Annu. Rev. Astron. Astrophys.* **35**, 1–32 (1997).

Evolutionary biology

A star is born

Stephen R. Palumbi

To see the constellations of Australia’s temperate east coast, you should look down instead of up. There, among the cobbles and kelp, live a swarm of multi-armed and multi-hued starfish of the genus *Patiriella*. The different species vary in size from a tea-cup saucer to a thumb-nail, but their most fascinating differences lie in how they reproduce^{1,2}.

Some produce tiny eggs that develop first into flop-limbed, free-swimming larvae before transforming into tiny crawling stars. Others produce larger, yolky eggs that develop directly into starfish with no intervening larval phase. Some adults brood their embryos in the gonad where cannibalistic juveniles fight their siblings for the right to be

born; others cast their eggs anonymously into the sea. Yet all these species are closely related, and a study by Hart *et al.*³, just published in *Evolution*, uses phylogenetic methods to reconstruct how this wealth of developmental diversity has evolved.

Developmental and evolutionary biology have had a very long flirtation. The stages of embryo development revealed by nineteenth-century microscopists were tantalizingly compared to changes in fossil life unearthed in progressive strata, and much was made of attempts to understand the rules by which development changed over evolutionary time. A common generalization from the early 1800s (often attributed to von Baer; see ref. 4) had it that the initial

phases of development are more refractory to evolutionary change than later stages, and that evolutionary stages are sculpted into a linear developmental sequence. Today, evolution of development is increasingly studied in the context of patterns of gene expression⁵ and the DNA sequences of gene control ‘switch-boxes’⁶. But the comparative approach remains crucial, and Hart and colleagues’ study of the evolution of developmental patterns in starfish overturns older generalizations about how such programmes evolve.

There are two big surprises in their results. The first is that, unexpectedly, there is no incremental progression from one developmental type to another. Based upon solid reasoning, Strathmann⁷ proposed that it is more difficult for complex larval structures to be gained than lost during evolution. Feeding larvae need a great deal of complicated machinery to catch and digest food, and Strathmann argued that loss of these structures to give rise to non-feeding, couch-potato larvae is more likely than the convergent evolution of feeding structures from non-feeding types. So developmental modes should more often go from complex, feeding larval forms to morphologically simpler, non-feeding types. More complex elaborations⁸ of this simple principle suggest that adult and larval characteristics might commonly evolve in stages such as loss of larval feeding, followed by loss of planktonic dispersal, followed by gain of parental brood protection and viviparity.

Hart *et al.*³ compared eight species of *Patiriella* and four species of the closely related genus *Asterina* by constructing a phylogeny based on mitochondrial DNA sequences,

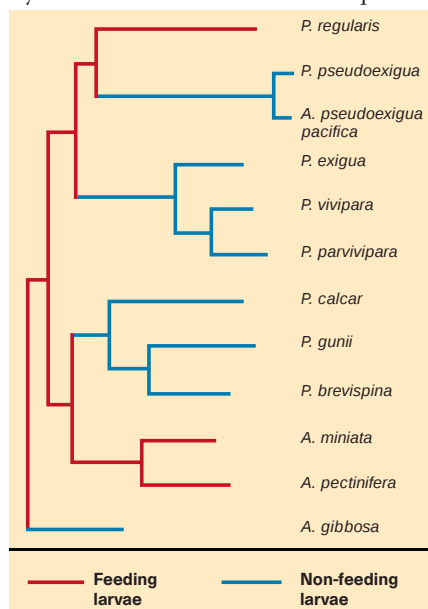


Figure 1 Phylogenetic relationships among 12 species of the starfish genera *Patiriella* and *Asterina*. As discussed in the text, Hart *et al.*³ find that non-feeding larval modes have evolved at least three times. (Redrawn from ref. 3.)

and tracing on it the different reproductive modes exhibited by these starfish. They "could not find an example of [the] transformation series", even though all the intermediate stages exist among the species they looked at. Strathmann's principle seems satisfied: non-feeding larval types are derived from ancestral feeding types. But Hart *et al.* found that evolution of non-feeding strategies has occurred at least three times independently within this single genus, and that all species with non-feeding larvae are not close relatives (Fig. 1). Moreover, species with benthic, non-feeding larvae, brood protection and viviparous adults evolve without evidence of the expected intermediate stages even in these closely related species.

This speed of developmental change is the second surprise, and probably explains the lack of intermediates. Although we don't have a well-calibrated molecular clock for these comparisons, the species concerned probably diverged within the past five to ten million years. During this short time there have been at least 16 major shifts in developmental mode. Moreover, shifts in early developmental characters are just as common as shifts in adult characters, contradicting the assertion that the early stages of development are less likely to change than later stages.

Hart and colleagues' work provides a kind of Hertzprung–Russell diagram for the development of biological stars. Other studies of marine invertebrates have also taken advantage of the increasingly well-described developmental variation within families or genera to understand how development is moulded by evolution^{9–12}. This research, which includes an intensive analysis of two species of sea urchin with contrasting developmental modes by Raff and colleagues (reviewed in ref. 12), also suggests that early developmental patterns are evolutionarily labile.

If early developmental events are not evolutionarily sacrosanct, are there any rules at all for the evolution of developmental systems? Some new rules have been suggested by a shift from a linear to a modular view of development. Developmental modules are still somewhat poorly defined, but broadly they represent complexes of interacting developmental instructions¹³. Evolution might proceed by shifting of modules in early development, or in shifting the relationships among existing modules. Raff¹³ has suggested that, instead of von Baer's linear laws, developmental rules during early and late stages are widely malleable, and that each lineage passes through a critical intermediate stage with less developmental flexibility. Developmental shifts early in embryology involve changes in early modules, and are permissible as long as they lead eventually to this critical 'phylogenetic' stage¹³.

Understanding the role of modules and phylotypic stages in the evolution of development will require comparisons of many related species with different developmental modes, and unravelling of the molecular networks involved. These multi-taxa analyses are made possible by the advent of rigorous molecular systematic methods for comparing species (such as the *Patiriella* starfish) never before brought to the genetics laboratory. The approach also places evolutionary changes in a distinct phylogenetic framework in which the direction of evolutionary changes can be discerned if enough taxa are compared. In this way, the wealth of developmental variation within genera and families, especially among thousands of species of marine invertebrates, can be used to help satisfy the developmental biologist's long-standing fascination with evolution. □

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1. Byrne, M. *Mar. Biol.* **114**, 297–316 (1992).
2. Byrne, M. *Mar. Biol.* **125**, 551–567 (1996).
3. Hart, M., Byrne, M. & Smith, M. J. *Evolution* **51**, 1848–1861 (1997).
4. Ghiselin, M. *Metaphysics and the Origin of Species* (State Univ. NY Press, Albany, 1997).
5. Lowe, C. J. & Wray, G. A. *Nature* **389**, 718–721 (1997).
6. Yuh, C.-H. & Davidson, E. *Development* **122**, 1069–1082 (1996).
7. Strathmann, R. *Evolution* **32**, 894–906 (1978).
8. McEdward, L. & Janies, D. A. *Biol. Bull.* **184**, 255–268 (1993).
9. Reid, D., Rumbak, E. & Thomas, R. *Phil. Trans. R. Soc. Lond. B* **351**, 877–895 (1996).
10. Hadfield, K., Swalla, B. & Jeffery, W. J. *Mol. Evol.* **40**, 413–427 (1995).
11. Arndt, A., Marquez, C., Lambert, P. & Smith, M. *Mol. Phylog. Evol.* **6**, 425–437 (1996).
12. Raff, R. A. *Dev. Biol.* **119**, 6–19 (1987).
13. Raff, R. A. *The Shape of Life* (Univ. Chicago Press, 1996).

Analgesia

The painless synergism of aspirin and opium

John T. Williams

The combination of aspirin and opioids is more analgesic than the summed effect of each drug given separately^{1,2} — in fact, this potent synergism has been used to obtain analgesia at doses where side-effects are minimal². The mechanism of this fortuitous interaction has been elusive, but Vaughan *et al.*³ (page 611 of this issue) now report that they have identified a synergistic synaptic interaction between opioids and non-steroidal anti-inflammatory drugs. The authors describe a mechanism of opioid action whereby activation of the μ -receptor causes a presynaptic inhibition of the trans-

mitter release that is mediated by arachidonic-acid metabolites (Fig. 1). In addition, the sensitivity of brain slices to opioids is markedly increased when they are pretreated with non-steroidal, anti-inflammatory analgesics.

The synergistic interaction between aspirin and opioids was proposed to occur because arachidonic acid is metabolized by two enzymatic pathways — through cyclooxygenase and lipoxygenase. So if cyclooxygenase is inhibited using non-steroidal anti-inflammatory analgesics, the lipoxygenase pathway will become the main

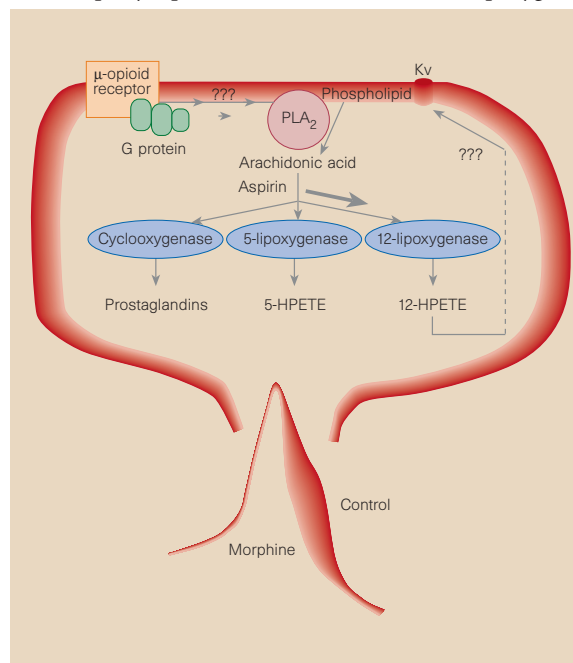


Figure 1 Schematic of a nerve terminal that releases GABA (γ -aminobutyric acid) in the midbrain periaqueductal grey. Activation of μ -opioid receptors results in the activation of phospholipase A₂ (PLA₂) by an unknown mechanism. Arachidonic acid, produced by the metabolism of phospholipids, can be further metabolized by the cyclooxygenase, 5-lipoxygenase or 12-lipoxygenase pathways. Metabolites of the 12-lipoxygenase pathway (including 12-hydroperoxyeicosatetraenoic acid; 12-HPETE) activate a voltage-dependent potassium conductance that decreases the duration of the action potential, resulting in an inhibition of transmitter release.

metabolic pathway. Not only will there be a decrease in the pain-producing prostaglandins (by inhibition of cyclooxygenase), but there will be increased production of pain-relieving lipoxigenase metabolites, which are activated by opioids.

Vaughan *et al.* used brain slices containing the periaqueductal grey (PAG) — a central site in the modulation of pain pathways⁴. One of the main effects of opioids in the PAG (and the measure used in this study) is the presynaptic inhibition of GABA (γ -aminobutyric acid)-mediated synaptic currents⁵. Although opioid-mediated presynaptic inhibition, particularly from GABA-releasing terminals, is common in the central nervous system, there is no consensus on the ionic mechanism(s) and second-messenger pathways involved⁶. Possible mechanisms to account for the presynaptic actions of opioids are the inhibition of voltage-dependent calcium currents and the activation of an inwardly rectifying potassium conductance.

The new study indicates that neither of these mechanisms is significant at this synapse in the PAG, but that opioids activate a voltage-dependent potassium current. (Such a mechanism has been described in cell bodies of isolated hippocampal neurons⁷.) The presynaptic inhibition by opioids in the PAG was reduced by 4-aminopyridine (4-AP) and dendrotoxin, both of which block voltage-dependent potassium conductances. In the same cells, the presynaptic inhibition caused by activation of GABA_B receptors with baclofen was not sensitive to 4-AP or dendrotoxin. This indicates selectivity of the opioid action. In another study⁸, activation of a similar voltage-dependent potassium conductance in sensory neurons of *Aplysia* decreased both the duration of the action potential and the release of transmitter. This mechanism could account for the opioid-mediated inhibition of the GABA release that is induced by action potentials. But the decrease in the frequency of calcium-independent miniature GABA-mediated synaptic currents by opioids was also sensitive to 4-AP and dendrotoxin.

Opioid inhibition of these currents in the PAG was blocked by inhibitors of arachidonic-acid metabolism. This has not been seen before in mammals, although a similar mechanism has been shown to mediate presynaptic inhibition in sensory neurons in *Aplysia*⁸. Many of the enzyme inhibitors that were used in that study were also found by Vaughan *et al.* to be effective in the PAG. In both cases, the 12-lipoxygenase pathway generates compounds that effectively inhibit transmitter release. Identification of the specific metabolites will depend on potent and selective enzyme inhibitors, because exogenous arachidonic acid and metabolites in brain slices are limited by slow diffusion

and metabolic instability. A critical remaining question is the connection between opioid receptors and the activation of phospholipase A₂, although an indirect link has been shown in expression systems⁹.

There was a striking increase in the sensitivity of brain slices to opioids after they were treated with inhibitors of cyclooxygenase or 5-lipoxygenase. Morphine, for example, caused a weak inhibition when applied alone, but in the presence of indomethacin, aspirin or caffeic acid it produced a near-maximal inhibition. Vaughan *et al.* suggest that the increased sensitivity to opioids resulted from an increased availability of arachidonic acid, such that metabolism through the 12-lipoxygenase pathway was increased. This mechanism may explain the synergism — rather than simple, additive interactions — between aspirin and opioids in the central nervous system.

A synergistic interaction between non-steroidal, anti-inflammatory analgesics and both μ -opioid and $\alpha 2$ -adrenoceptor agonists, but not κ -opioid or adenosine-A1 receptor agonists, has already been convincingly shown using intrathecal administration in conscious, active animals¹⁰. So this interaction is selective but not exclusive.

Because activation of both the μ - and $\alpha 2$ -receptors causes potent inhibition of transmitter release in the dorsal horn of the spinal cord, studies at the cellular level will be critical to test the hypothesis that has been put forward from this work in the PAG. If this cellular interaction is a common mechanism, identification of arachidonic-acid metabolites, selective enzyme inhibitors and agonists at other receptors (such as the $\alpha 2$ -adrenoceptor) may result in safe and effective treatments for pain. □

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1. Maves, T. J., Pechman, P. S., Meller, S. T. & Gebhart, G. F. *Anesthesiology* **80**, 1094–1101 (1994).
2. Beaver, W. T. *Am. J. Med.* **77**, 38–53 (1984).
3. Vaughan, C. W., Ingram, S. L., Connor, M. A. & Christie, M. J. *Nature* **390**, 611–614 (1997).
4. Morgan, M. M., Heinricher, M. M. & Fields, H. L. *Neuroscience* **47**, 863–871 (1992).
5. Vaughan, C. W. & Christie, M. J. *J. Physiol. (Lond.)* **498**, 463–472 (1997).
6. Cohen, G. A., Doze, V. A. & Madison, D. V. *Neuron* **9**, 325–335 (1992).
7. Wimpey, T. L. & Chavkin, C. *Neuron* **6**, 281–289 (1991).
8. Piomelli, D. *et al. Nature* **328**, 38–43 (1987).
9. Fukuda, K., Kato, S., Morikawa, H., Shoda, T. & Mori, K. *J. Neurochem.* **67**, 1309–1316 (1996).
10. Malmberg, A. B. & Yaksh, T. L. *Anesthesiology* **79**, 211–213 (1993).

Deafness

Sounds from the cochlea

Karen B. Avraham

To be defective in language, for a human being, is one of the most desperate of calamities, for it is only through language that we enter fully into our human estate and culture, communicate freely with our fellows, acquire and share information. (Oliver Sacks; ref. 1)

Language can take on many forms — from signs or gestures, to words produced by vocal cords. The inner ear is fundamental for the latter, enabling us to grasp not only the words but non-verbal communication as well, including laughter and music. Our understanding of the molecular basis of inner-ear function has increased dramatically in past years with advances in gene mapping and cloning. Now, a report by Lynch *et al.*² in *Science* describes the identification of a gene that is defective in a family with profound progressive hearing loss. And in this month's issue of *Nature Genetics*, Everett *et al.*³ report that they have isolated the gene for Pendred syndrome, an inherited disorder of congenital deafness and thyroid disease. These discoveries pave the way for the dissection of two major cellular pathways, actin polymerization and sulphate transport, in the inner ear.

In the Western world, 0.1–0.2 per cent of

children are born deaf, with this number reaching a higher proportion in isolated communities throughout the world. Nearly half the population may lose part or all of their ability to hear by the age of 80. Hearing loss can occur prelingually (before speech development) due to genetic defects or childhood diseases, or postlingually due to inherited gene mutations, environmental noise pollution, infection or ageing. Hearing impairment may occur in association with other symptoms, in the form of syndromic deafness, or as an isolated finding (non-syn-

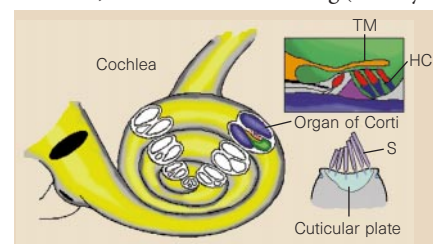


Figure 1 Cross-section of the cochlea — the portion of the inner ear responsible for hearing. The upper right figure shows an enlargement of the organ of Corti containing the tectorial membrane (TM) and hair cells (HC); the lower right figure shows stereocilia (S) projecting from the cuticular plate on the apical surface of a hair cell.

dromic deafness, accounting for about 70 per cent of hearing-loss cases).

Scientists in Mary-Claire King's laboratory, with Pedro Leon in Costa Rica, have now found² that the deafness locus *DFNA1* in the large Costa Rican family 'M' encodes diaphanous, a member of the formin family of proteins. Diaphanous is involved in cytokinesis and cell polarity, and homologues have been identified in *Drosophila*, yeast and mice. Diaphanous recruits profilin — an actin-monomer binding protein — to the membrane, where it promotes actin polymerization in a GTP-bound, Rho-dependent manner. In family M, diaphanous has a carboxy-terminal truncation and, although the protein is broadly expressed in human tissues, the only site of damage is in the sensory hair cells of the cochlea (Fig. 1). This implies a specialized role for diaphanous in hair cells.

Hair cells possess a rich cytoskeleton meshwork. They also have stereocilia which respond to sound, leading to membrane depolarization and subsequent neurotransmitter release. Mutations in diaphanous may affect the dynamic actin polymerization that occurs during the amplification of sound reception, organization of the cuticular plate (an actin-rich structure into which the stereocilia are inserted), and/or reordering of the paracrystalline array of actin filaments in the stereocilia.

Everett *et al.*³ isolated the Pendred syndrome gene, *PDS*, taking advantage of the Human Genome Project's systematic sequencing of human chromosome 7 at the Washington University Genome Sequencing Center. (Data are deposited in the database regularly, and are available on the Web at <http://genome.wustl.edu/gsc>, offering a tremendous short-cut in positional candidate cloning.) Patients with Pendred syndrome suffer from goitre, which is often variable in its expression, and deafness. Their cochleas contain fewer turns than normal, and no (or few) hair cells.

The *PDS* gene encodes a putative sulphate transporter protein called pendrin. The transport of SO_4^{2-} groups is required for sulphation, which is intrinsic to modification of proteoglycan glycosaminoglycan side chains and is one of the major post-translational modifications of tyrosine residues. Pendred syndrome may affect the thyroid because the main secretory product of thyroid follicular cells, thyroglobulin, requires sulphation. And in the inner ear, sulphation is needed for functioning of components involved in the innervation, structural development and/or function of auditory transduction — these include growth factors and the tectorial membrane of the organ of Corti. Alternatively, because thyroid hormones are known to be vital for development of the inner ear, the cochlear defects seen in patients with Pendred syndrome may be

secondary to thyroid dysfunction.

This has been a tremendously exciting year for the genetics of hearing loss. Mutations in connexin 26 (*CX26*) were discovered in non-syndromic hearing loss⁴, and were subsequently found to be prevalent in deaf populations^{5–7}. Given the ease in screening for mutations in *CX26* (the gene is encoded by a single exon), the implications for screening and genetic counselling for the hearing-impaired are dramatic. Myosin VIIA (*MYO7A*), which was originally found to cause deafness and retinitis pigmentosa in Usher's syndrome type IB and deafness in the mouse mutant shaker-1, has now been shown to cause non-syndromic recessive and dominant hearing loss as well^{8,9}. Moreover, two genes encoding proteins that are known to interact with each other — IsK (a membrane-spanning glycoprotein) and KvLQT1 (a potassium channel) — are involved in Jervell and Lange-Nielsen syndrome^{10–12}. People with this rare form of syndromic deafness also suffer from fainting spells caused by abnormal ventricular repolarization, which may result in early death. But care must be taken when identifying mutations. One of the original *CX26* variants identified in profoundly deaf siblings from a

family with dominant hearing loss may represent a simple polymorphism, because this variant has been identified in people with normal hearing¹³.

The list of genes involved in deafness that have been discovered in the past year alone points to an amazingly complex repertoire of proteins in the inner ear. Dissecting the functions of these proteins will provide an enormous amount of information regarding their normal roles in the cochlea, and may eventually help to alleviate hearing loss of all forms. □

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1. Sacks, O. *Seeing Voices* (Harper Collins, New York, 1989).
2. Lynch, E. D. *et al. Science* **278**, 1315–1318 (1997).
3. Everett, L. A. *et al. Nature Genet.* **17**, 411–422 (1997).
4. Kelsell, D. *et al. Nature* **387**, 80–83 (1997).
5. Zelante, L. *et al. Hum. Mol. Genet.* **6**, 1605–1609 (1997).
6. Denoyelle, F. *et al. Hum. Mol. Genet.* **6**, 2173–2177 (1997).
7. Carrasquillo, M. M., Zlotogora, J., Barges, S. & Chakravarti, A. *Hum. Mol. Genet.* **6**, 2163–2172 (1997).
8. Liu, X.-Z. *et al. Nature Genet.* **16**, 188–190 (1997).
9. Liu, X.-Z. *et al. Nature Genet.* **17**, 268–269 (1997).
10. Neyroud, N. *et al. Nature Genet.* **15**, 186–189 (1997).
11. Schulze-Bahr, E. *et al. Nature Genet.* **17**, 267–268 (1997).
12. Tyson, J. *et al. Hum. Mol. Genet.* **6**, 2179–2185 (1997).
13. Scott, D. A., Kraft, M. L., Stone, E. M., Sheffield, V. C. & Smith, R. J. H. *Nature* (in the press).

Anticancer drugs

Ringing the changes

Andrew Holmes

An increasing problem in cancer chemotherapy is multiple drug resistance. To fight it, medicinal chemists are continually on the lookout for new cytotoxic compounds, and they have allied themselves with specialists in natural products, synthesis and high-throughput screening. The discovery of the powerful anticancer properties of taxol* is a tribute to this multidisciplinary approach to cancer chemotherapy. Taxol has been followed by discodermolide, the epothilones and, in a patent application, eleutherobin. These all work by promoting the formation of stable bundles of microtubules, and so inhibiting cell division. (Indeed, a routine screening method for new compounds is to measure their binding to the 'taxol-binding domains' of microtubule assemblies.) The newest member of this family is eleutherobin, whose isolation and microtubule-stabilizing properties were announced in September¹. Astonishingly, that has already been followed by the compound's total synthesis and the determination of its absolute stereochemistry, described in two papers from Nicolaou *et al.*^{2,3}.

The structural diversity of these successive generations of 'taxol mimics' is quite remarkable, but they have all now succumbed to syn-

thesis^{2–4}. Eleutherobin and sarcodictyin A (ref. 5) are members of the family of 1,4-oxa-cladiellanes, including the anti-inflammatory valdivones⁶ and the eleuthosides⁷. Embedded in the tricyclic core of these structures (Fig. 1) is a medium-sized ring which can be

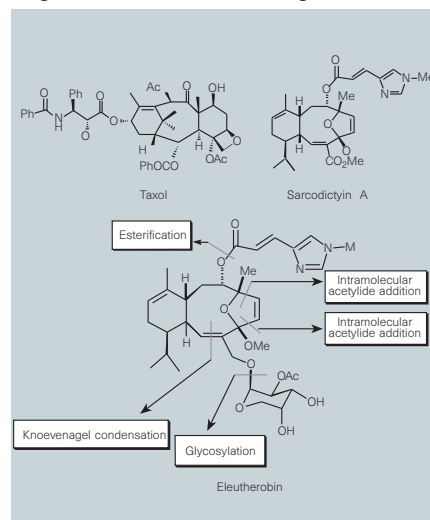


Figure 1 The anticancer drug taxol and the new taxol mimics eleutherobin and sarcodictyin A. Marked on eleutherobin are points where the tricyclic core of eleutherobin or sarcodictyin A can be easily broken. This hints at strategies for synthesis, using components derived from 'breaking bonds' at these points.

*Bristol-Myers Squibb has registered Taxol as a trademark and wishes the scientific community to use the name paclitaxel.

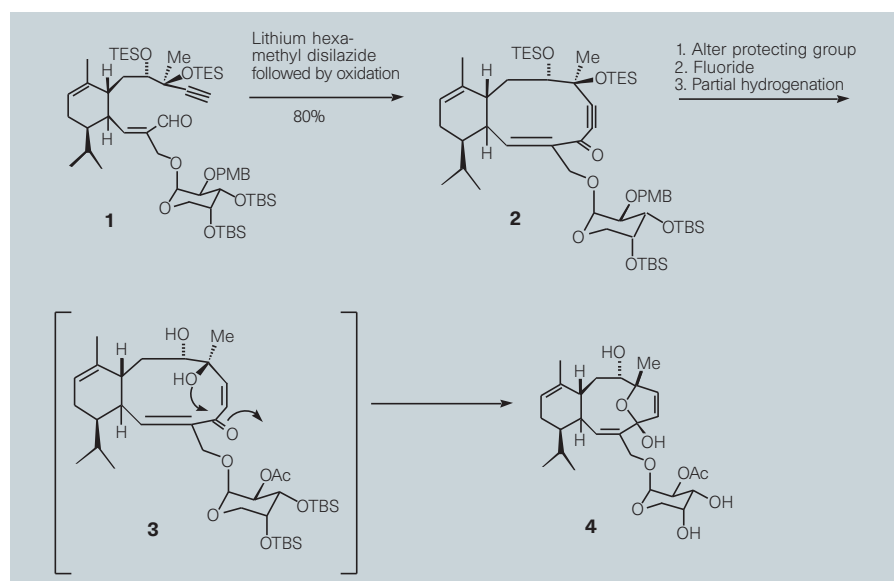


Figure 2 Closure of the ring of eleutherobin and formation of the 1,4-oxygen bridge.

viewed either as a 1,4-oxygen-bridged ten-membered ring or as a nine-membered oxacycle bridged by a double bond across the 2,9-positions. An alternative oxygen bridging pattern is seen in eunicellin⁸. Eleutherobin differs from sarcodictyin A largely in the detail of the ring substituents and the presence of a β -linked 2''-O-acetyl-D-arabinose, which is hard to synthesize.

Although the closure of medium-sized rings from open-chain precursors is usually a daunting task, it can be much easier when the two ends to be closed are pre-oriented (for example, by attachment to adjacent positions on a rigid six-membered ring). This is the strategy adopted by Nicolaou and co-workers in preparing the tricyclic core of eleutherobin and sarcodictyin A. Their disconnection strategy is summarized in Fig. 1. This synthesis yields the tricyclic skeleton in just a few steps. The difficult introduction of the glycosidic side-chain substituent was executed before formation of the medium ring, and the *N*(6')-methylurocanic acid was attached at a late stage.

The important steps for the formation of the tricyclic core are shown in Fig. 2. The

acetylenic aldehyde 1 was built up in a stepwise fashion from (+)-carvone. The ten-membered ring was formed by intramolecular attack on the acetylenic aldehyde 1, followed by immediate oxidation to form the ketone 2. The oxygen closure reaction employed a bold intramolecular cyclization of the hydroxyketone 3 to produce an apparently stable hemiacetal 4, which could then be converted into the corresponding mixed methoxy acetal. The method has the advantage that various simple analogues can easily be prepared by attaching side-chain substituents to a relatively accessible tricyclic core. Eleutherobin and two such analogues all had tubulin polymerization activity comparable to taxol.

Novel structures will continue to emerge from the seemingly bottomless pool of secondary metabolites found in nature (the 1,4-oxacladiellanes were isolated from coral; Fig. 3). Advanced screening methods will enable biologists to determine their therapeutic potential, and the ingenuity of organic synthetic chemists will provide a rapid supply of compounds for further evaluation. Ultimately, of course, these drugs have to prove effective in human clinical trials, and it is simply too early to say whether eleutherobin will reach this stage. □

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Figure 3 A soft coral of the species *Eleutherobia*, from which the promising anticancer drug eleutherobin was isolated. The drug has now been synthesized. (Reproduced from *Tropical Pacific Invertebrates*, Coral Reef Press.)

1. Lindel, T. et al. *J. Am. Chem. Soc.* **119**, 8744–8745 (1997).
2. Nicolaou, K. C. et al. *Angew. Chem. Int. Ed. Engl.* **36**, 2520–2524 (1997).
3. Nicolaou, K. C. et al. *J. Am. Chem. Soc.* **119**, 11353–11354 (1997).
4. Cowden, C. J. & Paterson, I. *Nature* **387**, 238–239 (1997).
5. D'Ambrosio, M., Guerriero, A. & Pietra, F. *Helv. Chim. Acta* **70**, 2019–2027 (1987); **71**, 964–976 (1988).
6. Lin, Y., Bewley, C. A. & Faulkner, D. J. *Tetrahedron* **49**, 7977–7984 (1993).
7. Ketzinel, S., Rudi, A., Schleyer, M., Benayahu, Y. & Kashman, J. *J. Nat. Prod.* **59**, 873–875 (1996).
8. Macmillan, D. W. C. & Overman, L. E. *J. Am. Chem. Soc.* **117**, 10391–10392 (1995).

Daedalus

Payment in advance

Why are most pleasurable drugs illegal? Daedalus suspects the puritan conscience at work. It holds that pleasure is sinful, and should be followed by pain: indeed ideally it should be preceded and accompanied by it, too. Thus traditional Western morality tolerates sexual pleasure, as long as it is soured with guilt and followed by pregnancy; alcohol is accepted because the subsequent hangover squares the account. But amphetamines, marijuana and the hard drugs dodge this dismal retribution. Hence their illegality — which also provides the hard drugs with their own punishment, withdrawal symptoms if the user's costly illegal supply dries up. Daedalus is now inventing a drug matched to the puritan conscience. His vision is that of an alcohol which gives you the hangover first.

Puritans, advocating hard work and self-sacrifice for a distant and uncertain reward, should welcome the new product. Pleasure-seekers could only enjoy it by developing socially useful traits such as foresight and persistence. Its chemistry, however, could be tricky. Daedalus recalled that the 'congeners' in alcoholic drinks, alleged to be responsible for the hangover, are often esters of some kind. So a suitable complex ester of ethyl alcohol might be arranged to hydrolyse very slowly in the digestive tract, releasing alcohol into the body after an initial hangover had begun to fade. Various opiate molecules might similarly be bound in unpleasant form, to be gradually released in belated recompense for the initial misery they inflict. But he then mused that the tricyclic and SSRI antidepressants also take a long time to start cheering the patient up. Their initial effects can be encouragingly distressing.

DREADCO pharmacologists are now exploring these avenues. With luck, a DREADCO 'Pain and Pleasure Pill' will prove to be feasible and safe. The PPPill, as the company's ad-men are already calling it, will challenge the puritan conscience in its central tenets. Will it be banned or welcomed? If the latter, Daedalus sees it as a potential aid to disgruntled industrial workers. He is planning its mild initial 'hangover' to last about a working shift. Its user will take a PPPill just before the shift begins, creating a grim, joyless mood in which work is almost a relief. But as the shift ends, its delayed rewards will start to come through. The user will walk out of the works in a growing glow of rebound happiness.

David Jones

The past and the future of El Niño

Peter J. Webster and Timothy N. Palmer

If events run true to form, the present El Niño is approaching a climax — which is why, acknowledging a Christmas connection, the phenomenon was so named. Predicting these events and their consequences is a daunting task, but there is progress to report.

Over the past few months, the arrival of an El Niño event has never been out of the news. Put simply, this phenomenon is a warming of the upper Pacific Ocean, but it has global effects and has been blamed for just about every climatic and social upset in the second half of 1997. There have been claims that the current El Niño may be the strongest of this century and the harbinger of stronger events that might accompany climate change. But how well do we really understand this phenomenon?

El Niños

In general, the tropical Pacific Ocean is characterized by warm surface water (29–30°C) in the west but much cooler temperatures in the east (22–24°C). The body of warm water in the west is known as the Pacific 'warm pool', and it is associated with intense rainfall and the largest region of atmospheric heating². The warm pool is relatively deep, with the temperature decreasing slowly with depth to the thermocline, the region of rapidly changing temperature some 100–200 m below the surface, before dropping off more rapidly. The cooler sea-surface temperatures (SSTs) in the eastern Pacific are the result of cold water upwelled from below, forced by the trade winds converging into the warm pool. Collectively, the east–west SST gradient and the associated easterly trade winds constitute a state of quasi-equilibrium for the ocean–atmosphere system³.

Roughly every three to seven years, this

state of quasi-equilibrium breaks down — ocean warming occurs across the entire basin, and it can last for a year or occasionally longer. This is an El Niño event⁴. Typically, the warm pool is displaced to the east, causing a shift in the major precipitation regions of the tropics and the disruption of normal climate patterns at higher latitudes. During the past two decades there have been a number of major warm events. In 1982–83, the then-strongest El Niño of the century occurred. In 1986 a weaker warm event ensued and lasted through 1987. A far weaker one occurred in 1992, but was untypical because it appeared to continue for a further two years. Usually, an opposite and cooler state of the tropical Pacific follows a year or so after an El Niño. This phase, known as La Niña, is marked by a distinct cooling in the eastern Pacific, and it too is associated with perturbations of the global climate. The warming and cooling phases are interspersed with the more common, normal, or quasi-equilibrium, state.

El Niño and La Niña depend jointly on oceanic and atmospheric processes, and are the result of the instability of the quasi-equilibrium state. The onset of an El Niño is often marked by a series of prolonged westerly wind bursts in the western Pacific, which persist for one to three weeks and replace the normally weak easterly winds over the warm pool⁵. The most important impact of these wind bursts is to severely perturb the upper ocean and excite the eastward propagation

of large-scale Kelvin waves — these waves, which have wavelengths of thousands of kilometres, have their maximum amplitude on the thermocline³. Their main effect is not to advect warm water from the warm pool to the cooler eastern Pacific, but to suppress the upwelling of cold water in the central and eastern basin².

El Niño is linked to the regular annual cycle of SSTs in the tropical Pacific but, instead of cooling early in the northern spring, during an event the eastern ocean continues to warm. This observation has led to the speculation that the instability that produces El Niño results from the inability of the warm pool to export enough heat each year so that its heat content increases in time. In this sense, the warm pool is preconditioned for El Niño, and the westerly wind bursts may serve as triggers to release the stored energy.

The 1997–98 El Niño

In the spring of this year, unprecedented surface warming occurred over the tropical eastern Pacific Ocean and in the subtropics to the north. The warming continued to develop at a rate far faster than is usual for an El Niño, so that in October the SST in the eastern Pacific was as much as 5 °C higher than normal (Fig. 1). The event could well turn out to be among the strongest to occur this century. It is an indication of the advances in forecasting that, as early as October and November last year, over six months before observations confirmed El Niño onset, predictions were made by a number of research groups using a new class of coupled ocean–atmosphere models⁶ (see box on page 564).

Early observational indications came in the form of the westerly wind bursts in late 1996 and early 1997, which extended farther to the east than in a normal year, along with the amplification and propagation of the subsurface temperature anomaly. In the months that followed, successive westerly wind bursts encroached further eastward as SSTs increased in the central Pacific.

Other preliminary signals were apparent

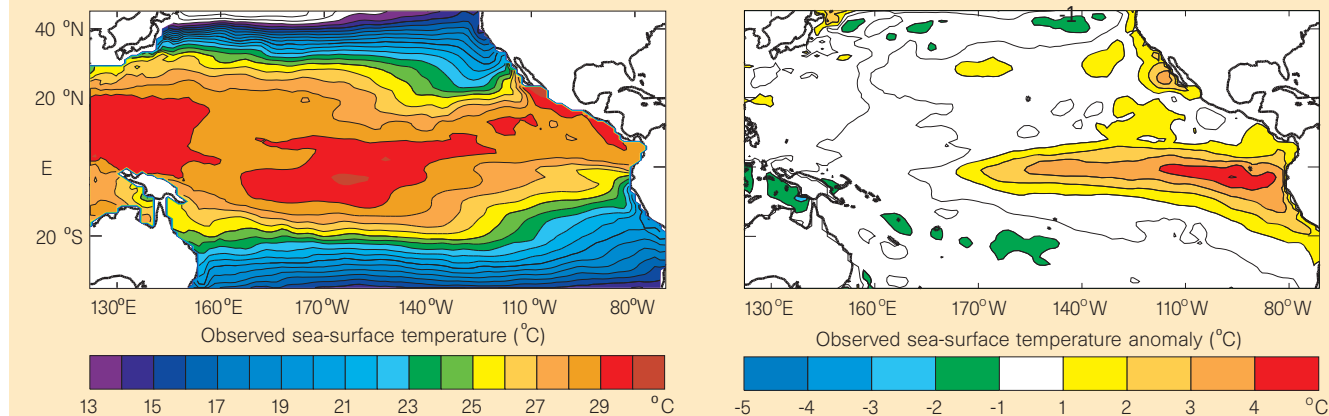


Figure 1 Left, Observed mean sea-surface temperature for October 1997. Right, Deviation of the October 1997 sea-surface temperature from the long-term (1980–95) October average. The warming in the eastern Pacific is more than 5 °C. (Data from ref. 19.)

in the subsurface structure of the Pacific, and became clear several months before surface warming was evident. In fact, the subsurface upper ocean had been warming well before the SST in the eastern Pacific had indicated that a major El Niño was under way (Fig. 2). Early in 1996 a 2–3 °C anomaly existed, indicating a moderate increase in the heat storage of the western Pacific warm pool. This anomaly persisted in the western Pacific throughout the year. During early 1997, the temperature anomaly slowly extended eastward, growing in intensity in the spring and progressively dominating the eastern Pacific Ocean; it finally surfaced in early summer. The subsurface temperature anomaly in the eastern Pacific is now in excess of 9 °C.

The consequences

El Niños are a regional phenomenon, but their 'footprint' is global. They are usually accompanied by severe drought over Australia and Indonesia, together with a weakened summer monsoon rainfall over South Asia⁷. Catastrophic flooding often occurs along the Pacific coast of South America and fish stocks disappear as ocean upwelling, containing high-nutrient cold water, diminishes. For societies where the impacts of El Niño are most direct, homes might be flooded, or destroyed by forest fire, and crops destroyed and fisheries ruined. Distinct disease patterns follow the waxing and waning of an event, through the contamination of water supplies by flooding and the creation of increased breeding areas for vectors such as mosquitoes. Water-borne diseases (hepatitis, dysentery, typhoid and cholera) have cycles associated with El Niño, as do vector-borne diseases (malaria, dengue and yellow fever, encephalitis, plague, hantavirus and schistosomiasis)⁸. El Niño also influences tropical cyclones, reducing their frequency in the Atlantic, but increasing it in parts of the Pacific.

Moreover, the climate of the extratropical regions remote from the Pacific may be affected by 'teleconnections' between tropical precipitation fields and large-scale circulation patterns in the extratropical atmosphere⁹. Teleconnections tend to change the probability of certain weather regimes in a particular region¹⁰. For example, the chance of stronger winter storms is greatly increased over southern California and the southern United States during El Niño. Beyond these severe regional impacts there is a broad influence on the global economy. Many of the commodities that are sensitive to El Niño, such as cereal crops, are traded on the world markets. Finally, the insurance industry is affected by the changing location of severe storms and hurricanes.

Cause and effect?

Can one say, however, that El Niños have 'caused' a particular perturbation to climate

in some part of the world remote from the tropical Pacific? How can they have a predictable impact, a season or more ahead, on a system whose day-to-day fluctuations are not predictable beyond a couple of weeks? The answers lie in the nonlinearity of the climate system — El Niño will not have a predictable influence on the instantaneous state of the atmosphere itself; rather, that influence falls on probabilities associated with the atmospheric state.

For example, El Niño introduces an asymmetry into the probability distribution of large-scale weather patterns, making some less likely and others more likely. Such patterns may correspond to periods of enhanced westerly winds or to more stagnant anticyclone conditions. Based on climatological records, one can associate probabilities with the occurrences of these weather patterns and, in this respect, in the extratropics, El Niño's effect is strongest over the north Pacific and North America¹⁰.

Further away, the influence of El Niño on weather patterns is generally statistically weaker, though certainly not negligible. In this sense, it is impossible to say that particularly severe weather in the extratropical latitudes has definitely been caused by El Niño.

But it is perfectly reasonable to ask whether the probability of such weather occurring is increased (or decreased) by El Niño. Take, for instance, two regions of the Northern Hemisphere, Europe and India, which this past summer experienced two different climate anomalies. Central Europe suffered devastating flooding associated with prolonged periods of rainfall. Over India, on the other hand, where the climatological links to El Niño are well established, monsoon rains were close to normal when drought was expected (J. Shukla, personal communication). Why?

As it happens, six-month coupled ocean-atmosphere ensemble forecasts, stemming from work at the institution of one of us (T.N.P.), did predict a statistically significant probability of higher than normal summer rainfall over parts of central and southern Europe⁶. Because these forecasts also correctly predicted the El Niño, and the Pacific SST anomalies were the largest anywhere in the globe, it is probable that the forecast of increased likelihood of central European rainfall was linked to El Niño. But a probability is only a probability — if an identical El Niño developed three or four years from now, we could not say with certainty that

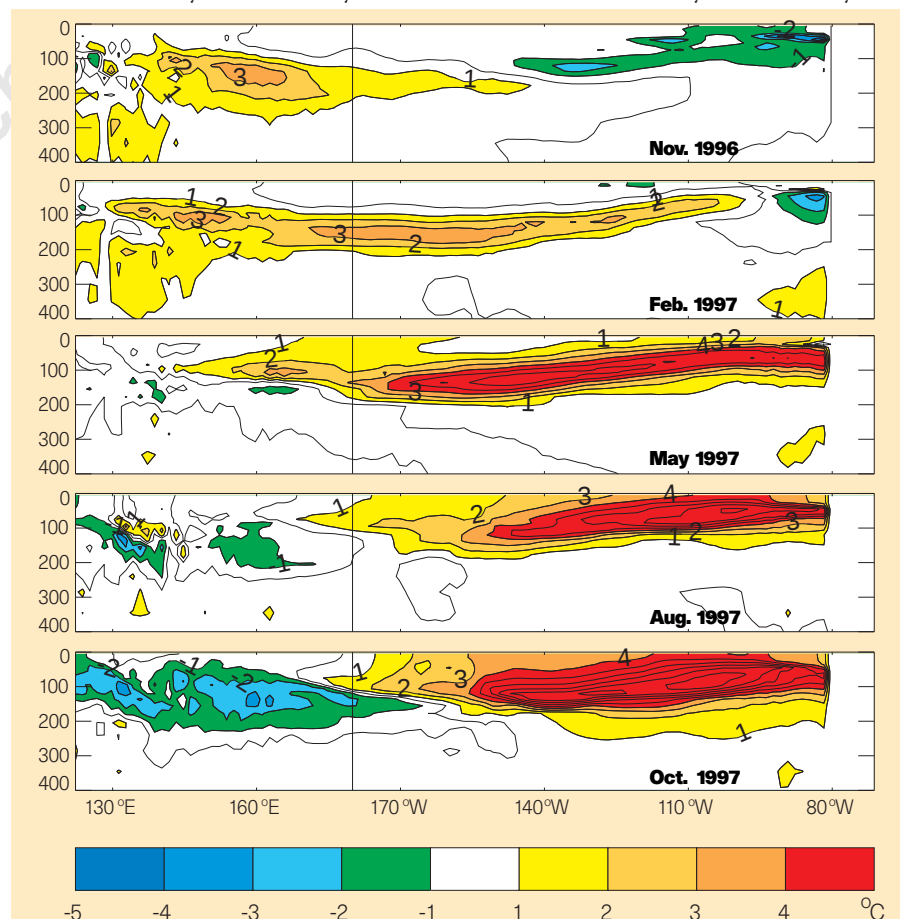


Figure 2 Cross-sections of ocean temperature along the Equator in the Pacific for November 1996, and February, May, August and October 1997. Contours show deviations from the long-term (1980–95) monthly mean distributions. Starting with a 3 °C anomaly in the western Pacific, the subsurface anomaly strengthens and moves eastwards during 1997, surfacing in the eastern Pacific in the northern spring. (Data from ref. 19.)

similar prolonged and heavy rain would occur.

The incidence of average, rather than below average, summer precipitation over India is equally intriguing. One school of thought holds that monsoons should be highly predictable¹¹. When the empirical relationship between Pacific SSTs and Indian rainfall fails, as it did this past summer, then it is presumed there are other precursor climate anomalies (such as the snow depth over the Eurasian continent during the spring season) that have conspired against El Niño to produce a normal monsoon. But there is a different view, one which we take¹². The Indian monsoon, like the extratropical circulations, has a chaotic component associated with an irregular alternation between periods of excessive rain or drought persisting from one to three weeks during a summer monsoon⁷. According to this

perspective, El Niño would influence the probabilities of these rainy and drought regimes, making the drought phase more likely and the active phase less likely. However, compared with the chaotic fluctuations, the influence of El Niño would not be strong enough to make the occurrence of a normal monsoon impossible during an El Niño year.

The outlook

The 1997–98 El Niño has been the best described and best modelled yet. It has been the first to be observed with the full network of moored Tropical Atmosphere–Ocean (TAO) buoys deployed to monitor atmospheric and ocean conditions between 135° E and 95° W and 10° N and 10° S (ref. 13). The flow of data stemming from these observational platforms, a system envisaged under the international Tropical Ocean–Global

Atmosphere (TOGA) programme¹⁴, has contributed to the success of forecasting the current event — which, after future analysis, will become the benchmark for major El Niños.

The 1997–98 event is expected to grow in intensity, reaching a maximum within the next few weeks. If it runs true to form, we may expect increased rainfall in the southwestern United States and on the Pacific coast of Peru and Ecuador; furthermore, the drought should continue over Indonesia and New Guinea, and become more pervasive over Australia. If the warm SST anomalies extend through the northern spring, there is an increased probability of poorer than average rainfall in South Asia in the summer of 1998, and maybe, some say, of above average rain over central Europe.

Many models are already predicting that the Pacific Ocean will move into the opposite phase, La Niña, later in 1998. These predictions will be a discriminating test of the new generation of forecast models, which in turn are being used to forecast global climate change. Success in evaluating natural climate fluctuations can give us confidence that such models will in time be able to meet another great challenge — quantitative prediction of the effects that mankind is having on the Earth's climate. □

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Models for prediction

A great deal of progress has been made in forecasting El Niños. For example, the event of 1982–83 was only evident once it had started. At that time, seasonal predictions were made using empirical models whose equations were based on statistical relationships derived from historical time-series data¹⁵. In 1986, however, Cane and Zebiak¹⁶ demonstrated the possibility of making very useful forecasts several seasons ahead, by taking the basic equations which describe Newton's laws of motion, together with the laws of thermodynamics, and applying them to describe the coupled dynamics of the ocean and atmosphere of the tropical Pacific. By the standards of comprehensive numerical weather-prediction models, the Cane–Zebiak model is relatively simple¹⁷. But, impressively, during the late 1980s and early 1990s it was used to predict the timing and magnitude of the

variations of sea-surface temperatures in the tropical Pacific (and hence El Niño).

Those successes led to the development of comprehensive ocean–atmosphere models, where the atmospheric component was either a numerical weather-prediction model, or a global climate model, and where the ocean component covered all of the ocean basins⁶. For a decade, it has been hard to prove that the comprehensive coupled models were better than the Cane–Zebiak model. That may have changed with the 1997–98 event, which comprehensive coupled models forecast whereas the Cane–Zebiak model did not.

Part of the answer may lie in the relatively simpler way that models of Cane–Zebiak complexity handle the data used to create the initial conditions for the ocean–atmosphere forecasts. Essentially, the comparatively large systematic errors of the simple models makes them harder to use

when assimilating these initial data. (Indeed, this was probably the case with the 1997–98 warming — later experiments have indicated that if a more comprehensive initial data set had been run on the Cane–Zebiak model, it too would have forecast a major El Niño with a similar lead time.) The comprehensive models also have such errors, but these have been reduced over the years and, in the long run, there is more scope to reduce them further than in the simpler models.

Empirical models can still be useful in prediction. Unlike the dynamically based models, however, their skill is obtained from statistical relationships derived from earlier, training data. Therein lies the rub, because on timescales of decades and centuries the world's climate is not stationary (which may or may not be due to anthropogenic effects)¹⁸, and that will always limit the predictive power of empirical models.

P.J.W. & T.N.P.

- Webster, P. J. & Lukas, R. *Bull. Am. Met. Soc.* **73**, 1377–1416 (1992).
- Webster, P. J. *Rev. Geophys.* **32**, 427–476 (1994).
- Philander, S. G. H. *El Niño, La Niña, and the Southern Oscillation* (Academic, New York, 1990).
- Trenberth, K. *Bull. Am. Met. Soc.* (in the press).
- Knox, R. & Halpern, D. *J. Mar. Res.* **40**, 329–339 (1982).
- Stockdale, T., Anderson, D., Alves, J. & Balmaseda, M. *Nature* (submitted).
- Webster, P. J. *et al.* *J. Geophys. Res.* (in the press).
- Epstein, P. *ENSO Signal* **2**, 1–3 (1995).
- Palmer, T. N. & Mansfield, D. A. Q. *J. R. Met. Soc.* **112**, 639–660 (1986).
- Ropelewski, C. F. & Halpert, M. S. *Mon. Weath. Rev.* **115**, 1606–1626 (1987).
- Charney, J. G. & Shukla, J. in *Monsoon Dynamics* (ed. Lighthill, J.) 99–110 (Cambridge Univ. Press, 1981).
- Palmer, T. N. *Proc. Ind. Natl. Sci. Acad.* **60**, 57–66 (1994).
- www.pmel.noaa.gov/toa/tao
- World Climate Research Programme *Scientific Plan for the Tropical Ocean–Global Atmosphere (TOGA) Programme WCRP Publ. No. 3* (World Met. Org., Geneva, 1995).
- Hastenrath, S. *Climate Dynamics of the Tropics* Ch. 9 (Kluwer, Dordrecht, 1994).
- Cane, M. A. & Zebiak, S. E. *Science* **228**, 1085–1087 (1985).
- Zebiak, S. E. & Cane, M. A. *Mon. Weath. Rev.* **115**, 2262–2278 (1987).
- Houghton, J. T. *et al.* (eds) *Climate Change 1995: The Science of Climate Change* (Cambridge Univ. Press, 1996).
- http://nic.fb4.noaa.gov/8000/research/cmb/climate_ocean.html

Callan's canyons

Jonathan Callan is a landscape artist with a difference. He is experimenting with the unpredictable by allowing his powdered medium to go with the flow, producing avalanches that create their own terrain.

Martin Kemp

Artists have always relied upon physico-chemical processes in the making of their works. The processes have ranged from the technological complexities of bronze casting to the suspension of pigments in various kinds of binding agents.

Traditionally, the behaviour of materials has needed to be understood and controlled, so that the potentialities of the various media might be exploited and their technical limits duly respected.

Recently, some artists have been prepared to endow process with a more dynamic and determining role, setting up situations in which the physical behaviour or chemical action of the medium is given its head. In effect, the parameters for the programme are established, but it is then left to run with results that are not subject to subsequent control and are not precisely predictable.

The young London artist, Jonathan Callan, has been exploring a range of processes, from the controlled modelling of phenomena in sculptural form to what may be described as 'free-style evolution of morphology'.

He is currently engaged upon a series of works that relies upon the properties of viscosity and flow in various semi-solid and granular materials. He is undertaking what he calls "material conversations". As he says, "to have a conversation with a nail you need a hammer".

In one series, of which the illustrated work is part, he begins with surfaces punctured by circular holes at irregular intervals. Granular material, in this case powdered cement, is then shaken evenly over the surface using a sieve. The piles that accumulate on the unperforated portions of the plastic 'stencil' are subject to a succession of avalanches.

The result, viewed laterally, is an extraordinary landscape of jagged peaks, hanging ridges, clustered summits and deep canyons. Seen from above, a cellular structure emerges, with a network of dividing walls surrounding the central spaces. The configuration obeys what he terms "a de-natured geological principle, of sedimentary deposits, the silting of a river estuary... a geography that seems both eminently 'natural' and highly 'artificial' — the Alps brand new".

Repeating the process with the same



Jonathan Callan, *Untitled* (1997), viewed from the side.

materials and surface configuration results in 'landscapes' that are similar with respect to some general rules — for example the highest 'mountains' correspond to the broadest portions of unperforated surface — but not perfect duplicates. The avalanches occur when the critical angle of the slope is exceeded, but the exact behaviour of successive landslides and their interaction with adjoining systems is not forecastable.

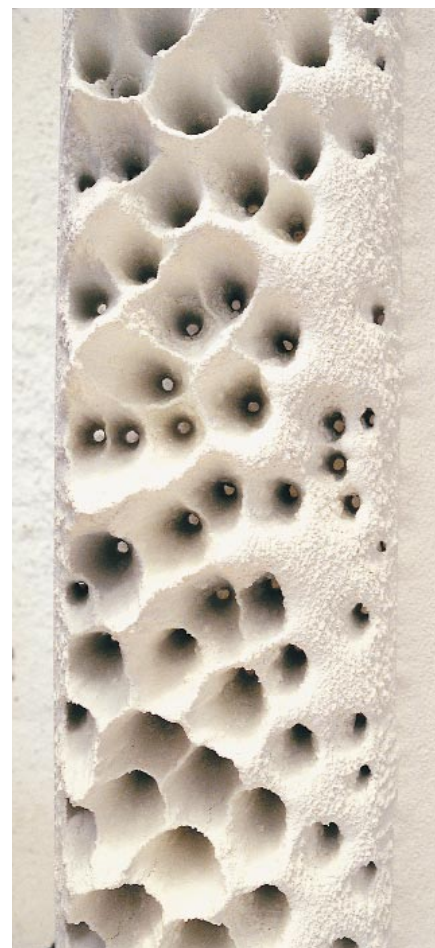
The same configuration of holes results in what we may describe as fraternal physiognomies rather than absolutely identical features.

For anyone concerned with developments in the sciences of complexity, Callan's dust piles will irresistibly recall the sandpile phenomenon that provided Per Bak, Chao Tang and Kurt Weisenfeld with their initial model of self-organized criticality.

Callan's 'experiments' are made with no knowledge of the scientific theory, yet they serve as elegant illustrations of what Bak has characterized as a "poised, critical state" of great delicacy in which tiny disturbances lead to catastrophic events.

The artist is not overtly concerned with the analysis of the process in the manner of a physicist, but has sensed artistically how such processes can result in topographies of timeless beauty. □

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Jonathan Callan, *Untitled* (1997), viewed from above.

An E2F-like repressor of transcription

The activity of a variety of genes whose products are involved in DNA replication and cell-cycle progression are regulated by E2F transcription factors, which bind to E2F sites on DNA in cooperation with members of the DP family of transcription factors¹. E2F sites can act as both positive and negative control elements². Transcriptional activation from E2F sites is mediated by 'free' E2F, whereas repression conventionally requires the formation of a complex with a pocket protein (retinoblastoma protein (RB), p107 or p130)³. Here we describe an E2F-like protein, termed EMA (for E2F-binding site modulating activity), which can act as a repressor of transcription without a pocket protein.

We identified EMA in a yeast two-hybrid screen with the DP-1 dimerization domain as a bait. The 2.4-kilobase mouse EMA complementary DNA contained a full-length open reading frame that translated into a protein of 272 residues (EMBL/GenBank database accession number AF032131). *In vitro* translation of the EMA cDNA results in a protein with a relative molecular mass of roughly 34,000 (34K) (data not shown). The EMA amino-acid sequence shares homology with E2F proteins (Fig. 1a), with features found conserved in all E2F family members — such as the DNA-binding and the dimerization domain — being highly conserved in EMA. However, the EMA protein lacks the activation domain found at the carboxy terminus of all known E2Fs and thus lacks the binding site for pocket proteins. Because EMA shows some but not all of the conserved sequence characteristics of E2Fs, EMA has to be considered as an E2F-like protein rather than a new member of the E2F family.

EMA binds DP-1 and DP-2 *in vitro*. In conjunction with DP-1, EMA recognizes a consensus E2F-binding site⁴ but shows a higher affinity for an E2F site containing a TCCCGCC core rather than a TCGCGCC core as present in the prototype E2F site⁵ derived from the E2 promoter (data not shown). When the high-affinity EMA-binding site is linked to a high 'basal' level promoter (pA10CAT-EMA; see legend to Fig. 1), co-transfection of EMA suppresses this promoter (Fig. 1b). This repression depends on the presence of a functional E2F-binding site because neither the control promoter (pA10CAT) nor a promoter containing a mutated E2F site (pA10CAT-EMAmut) were downregulated by EMA co-expression (Fig. 1b).

We found that the EMA protein contained an independent transcriptional repression domain⁶ that still functions when transferred to a heterologous DNA-binding

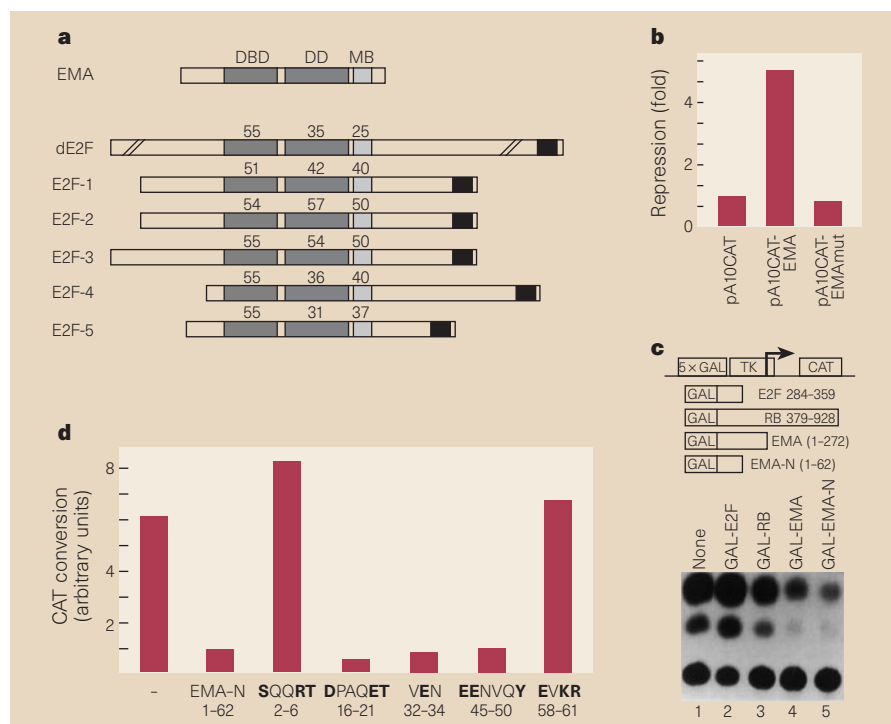


Figure 1 EMA is a transcriptional repressor. **a**, Schematic alignment of the mouse EMA protein and the different E2Fs isolated so far, including *Drosophila* E2F (dE2F). The percentage of identical residues within the DNA-binding domain (DBD), dimerization domain (DD) and marked box (MB) between EMA and each E2F family member is shown. The activation domain is shown in black. Yeast two-hybrid system was a gift from S. Hollenberg¹⁰. Full-length EMA cDNA clones were isolated from an adult mouse liver library (Stratagene). **b**, HeLa cells were transfected with a chloramphenicol acetyl-transferase (CAT) reporter construct containing the minimal SV40 early gene promoter (pA10CAT) linked to double wild-type (pA10CAT-EMA) or mutant (pA10CAT-EMAmut) EMA-binding sites. Repression is the ratio of control (pQ-Neo, Invitrogen) to EMA (pQ-HA-EMA) transfections. **c**, EMA contains an independent transcriptional repression domain. Reporter constructs contain five binding sites for the yeast transcriptional activator GAL4 next to the thymidine kinase gene promoter of the herpes simplex virus (TK). GAL4 fusion proteins contain sequences derived from E2F-1 (284–359), RB (379–928) and EMA (1–272 and 1–62) as indicated. **d**, Mutational analysis of the EMA N-terminal repression domain. Bold residues were each changed to alanines in individual groups as indicated. Numbers give the positions of the first and last residue of each group. All mutants were expressed to similar levels. Transient transfections, site-directed mutagenesis and western blots were done essentially as reported previously^{8,11}.

domain. A fusion protein of EMA and the GAL4 DNA-binding domain repressed the activity of the high 'basal' level promoter 5GALTK-CAT (ref. 7) more effectively than a GAL4-RB fusion⁸ (Fig. 1c). Because the EMA amino terminus but not the carboxy terminus is highly conserved between mouse and man (Anke Maassen and C. H., unpublished results) we tested whether this domain contains a repressive capacity. When the 62 N-terminal residues of EMA were fused to GAL4 this construct repressed transcription to an even greater extent than the full-length GAL-EMA fusion (Fig. 1c). This repression was not due to steric hindrance because a transcriptionally inert fragment of E2F-1 (ref. 9) had no significant effect on the activity of the 5GALTK-CAT reporter. Thus the EMA protein is an active transcriptional repressor with an indepen-

dent N-terminal repression domain.

The EMA N terminus shows no significant sequence similarity to any other protein in the database. Therefore, we specifically mutated residues throughout the EMA N terminus to define the repressor region further (Fig. 1d). Residues in the central part of the repression domain do not contribute significantly to the repressor activity. In contrast, mutations of either the N or C terminus of the repression domain completely abolish repressor activity. Thus, the N terminus of EMA contains two small domains that seem to cooperate in downregulation of transcription, possibly by serving as a docking site for co-repressor proteins.

The identification of EMA adds a further and unexpected twist to the already complicated regulatory network involving E2F/DP and pocket proteins. It remains to

be seen exactly what role EMA has in the regulation of E2F-response genes but we suggest that EMA represents the first member of a new class of E2F-like proteins acting to downregulate gene expression in a manner independent of pocket proteins.

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1. Beijersbergen, R. L. & Bernards, R. *Biochim. Biophys. Acta* **1287**, 103-120 (1996).
2. Weintraub, S. J. *et al. Nature* **358**, 259-261 (1992).
3. Weinberg, R. A. *Cell* **81**, 323-330 (1995).
4. Chittenden, T. *et al. Cell* **65**, 1073-1082 (1991).
5. Kovacs, L., Reichel, R. & Nevins, J. R. *Cell* **45**, 219-228 (1986).
6. Hanna Rose, W. & Hansen, U. *Trends Genet.* **12**, 229-234 (1996).
7. Yew, P. R. *et al. Genes Dev.* **8**, 190-202 (1994).
8. Weintraub, S. J. *et al. Nature* **375**, 812-815 (1995).
9. Hagemeier, C. *et al. Nucleic Acids Res.* **21**, 4998-5004 (1993).
10. Vojtek, A. B. *et al. Cell* **74**, 205-214 (1993).
11. Hagemeier, C. *et al. EMBO J.* **13**, 2897-2903 (1994).

Ribonuclease inhibits Kaposi's sarcoma

Kaposi's sarcoma (KS) is a cancer closely associated with AIDS. Crude, commercial human urinary chorionic gonadotrophin (hCG) preparations have previously been found to inhibit the growth of KS cell lines *in vitro* and in immunodeficient mice^{1,2}, and to induce tumour regression of KS lesions in AIDS patients^{3,4}. Here we report

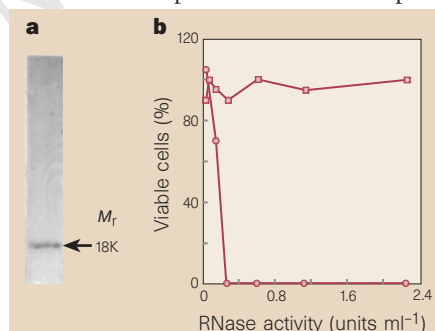


Figure 1 Analysis of RNase from hCG preparations. **a**, Purified RNase (20 ng protein) was subjected to SDS-PAGE on a Phast gel (gradient 10-15%; Pharmacia Biotech) and the gel stained with silver. **b**, Effect of the 18K RNase on viability of KS Y-1 (circles) or HeLa cells (squares). Cells (5×10^4 cells per well, 96-well tray) were incubated with purified RNase (100 μ l MDCB medium, Gibco; 16 h; 37 °C) and viability determined with WST-1 reagent (Boehringer-Mannheim).

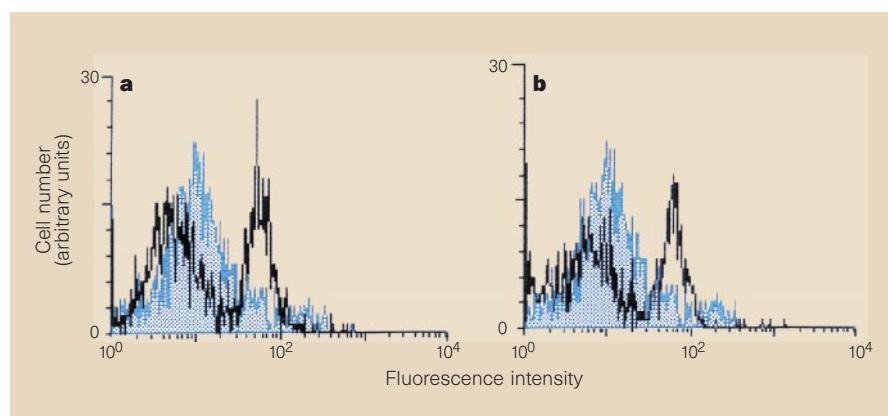


Figure 2 Induction of apoptosis in KS Y-1 cells by the 18K RNase. KS Y-1 cells (5×10^5 cells per well, 24-well tray) were incubated with purified RNase (**a**, 4.46 units RNase activity ml⁻¹; **b**, 1.12 units RNase activity ml⁻¹) in a total volume of 200 μ l MDCB medium (16 h, 37 °C) and assayed for apoptosis with the TUNEL reaction (Boehringer-Mannheim). Cells were analysed by FACS; induction of apoptosis (black-enclosed peaks) is shown by an increase in fluorescence intensity attributable to the binding of fluorescent antibody to apoptotic nuclei. Blue peaks are control incubations. Values are percentages of the reading for untreated cells.

the purification of a ribonuclease (RNase) from a commercial hCG preparation. The pure enzyme killed KS cells *in vitro*, apparently by apoptosis, suggesting that effects of commercial hCG preparations on KS cells and tumours are due, at least in part, to RNase present as a contaminant in these preparations.

We have shown a close association between a human urinary RNase (apparent relative molecular mass (M_r) 18,000) and the β -core fragment of hCG (ref. 5). During the present study we purified the RNase to electrophoretic homogeneity using gel permeation and ribonuclease inhibitor-affinity chromatographies (Fig. 1a; specific activity 892 units per mg of protein (ref. 6)). After electroblot procedures the pure RNase did not crossreact with monoclonal or polyclonal antibodies raised against hCG β -core protein, but it had potent dose-dependent killing activity against a KS Y-1 cell line¹ (Fig. 1b). Furthermore, the RNase induced apoptosis in the KS Y-1 cells in a dose-dependent manner (Fig. 2a). It had no effect on a HeLa cell line (Fig. 1b and data not shown).

Until now, anti-KS activity detected in commercial preparations of chorionic gonadotrophin has been attributed solely to hCG. The present results indicate that an RNase present in these crude preparations makes a major contribution to anti-KS activity. Indeed, the close association of the β -core of hCG with the RNase during purification procedures⁵ might explain why partly purified β -core preparations contain particularly potent anti-KS activity. In this regard, recent results obtained by Albini *et al.*² must be borne in mind. These authors found that recombinant hCG inhibited growth of KS cells and that the inhibitory effect was blocked by anti-hCG antibodies. It therefore seems likely

that at least some of the anti-KS activity detected in commercial hormone preparations is attributable to intact or fragmented hCG. Thus, overall potent anti-KS activity of commercial hCG preparations might result from the combined effects of hCG and RNase activities.

Amino-terminal sequence analysis of the 18K RNase present in human urine indicates that the enzyme belongs to the RNase superfamily that includes onconase^{5,7}. Onconase is an amphibian ribonuclease that is cytotoxic to cancer cells *in vitro* and exhibits anti-tumour activity in animal models⁸; it has shown promise in the treatment of human cancers and is currently in phase III clinical trials in patients with pancreatic carcinoma^{9,10}. The 18K human RNase might have a similar role in protection against neoplastic disease. It may prove possible to exploit the anti-neoplastic properties of this enzyme as part of a new regime for the treatment of AIDS-related KS and other tumours.

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1. Lunardi-Iskandar, Y. *et al. Nature* **375**, 64-68 (1995).
2. Albini, A. *et al. AIDS* **11**, 713-721 (1997).
3. Harris, P. J. *Lancet* **346**, 118-119 (1995).
4. Gill, P. S. *et al. N. Engl. J. Med.* **335**, 1261-1269 (1996).
5. Griffiths, S. J., Bramley, T. A., Menzies, G. S. & Adams, D. J. *Mol. Cell. Endocrinol.* **134**, 69-76 (1997).
6. Sakakibara, R., Hashida, K., Kitahara, T. & Ishiguro, M. *J. Biochem. (Tokyo)* **111**, 325-330 (1992).
7. Schein, C. H. *Nature Biotechnol.* **15**, 529-536 (1997).
8. Mikulski, S. M., Ardel, W., Shogen, K., Bernstein, E. H. & Menduke, H. J. *Natl. Cancer Inst.* **82**, 151-153 (1990).
9. Mikulski, S. M., Grossman, A. M., Carter, P. W., Shogen, K. & Costanzi, J. J. *Int. J. Oncol.* **3**, 57-64 (1993).
10. Newton, D. L. *et al. Protein Eng.* **10**, 463-470 (1997).

Mosaicism in Turner's syndrome

Skuse *et al.*¹ report that females with Turner's syndrome who have retained the paternal X chromosome (X^P) tend to achieve better cognition and social adjustment scores than those with the maternal X (X^M). As sex-chromosome mosaicism is frequent in Turner's syndrome, we argue that the presence of residual Y chromosomal sequences in the brain, which is exclusively possible in X^M Turner patients, might be a realistic alternative to the hypothesis of an imprinted X-linked locus as a reason for the behavioural differences between X^M and X^P Turner patients.

The authors present a fascinating genetic explanation for the sex dimorphism of vulnerability to developmental disorders of language and social cognition. They attribute the behavioural and cognitive differences between Turner's syndrome patients with maternally and paternally derived X chromosome (X^M or X^P) to a putative imprinted locus on the X chromosome. However, they do not address the fact that, with respect to the typical mechanism of postzygotic X or Y chromosome loss underlying the origin of Turner's syndrome², most Turner's syndrome patients are cytogenetic mosaics between a majority of 45,X cells (45 chromosomes per cell, with only one sex chromosome) and smaller clones of cells with either 46,XX, 46,XY or aberrant sex-chromosome complements^{3,4}. Held *et al.*⁵ even hypothesize that mosaicism in Turner's syndrome, which can arise only postzygotically, is a prerequisite for survival in early pregnancy. Clones of variant cells in Turner's syndrome patients often escape cytogenetic analysis but can be of great significance for the phenotype⁶. Chromosomal mosaics can be unevenly distributed between different organs within the same individual, and the analysis of blood lymphocytes alone has been shown to fail to detect mosaicism present in other tissues of the same Turner patient⁵. Thus, it is realistic to assume the presence of residual male or, in other cases, female cells in the brains of many Turner's syndrome patients.

X^P Turner patients form a homogeneous population as to the possible clones of normal cells but X^M Turner patients do not: X^P can only arise through the loss of a maternal X chromosome, whereas X^M can result from the loss of either the paternal X chromosome or the paternal Y chromosome. The origin of X^M Turner patients from both male and female zygotes is the reason why X^M is more common than X^P . Consequently, a

mosaic of cells containing Y-chromosomal genes (either as residual normal XY cells or as cells with marker chromosomes containing Y-chromosomal genes⁷) is possible only in X^M Turner patients. To us, the possible presence of cells with Y-chromosomal genes in the brains of patients from the X^M but not the X^P group is at least as convincing an explanation for their statistically more marked behavioural disabilities as the, admittedly elegant, hypothesis of X-chromosome imprinting.

The genetic heterogeneity of the X^M group might also explain the twice as high standard deviation of verbal IQ within the X^M compared with the X^P group. Even within the general population, females tend to show higher verbal ability than males⁸. Moreover, the exclusive possibility of XY mosaicism in X^M Turner patients matches perfectly with the fact that Skuse *et al.* found autism, which is observed mostly in males, only in X^M patients. It remains to be studied to what extent the postulated sex-chromosome imprinting contributes to the marked psychodevelopmental vulnerability of XYY males⁹, all of whom have an X^M chromosome, and of XXY Klinefelter's syndrome males, who can have either two X^M chromosomes or one X^M and one X^P .

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1. Skuse, D. H. *et al.* *Nature* **387**, 705–708 (1997).
2. Weiss, E., Loevy, H., Saunders, A., Pruzansky, S. & Rosenthal, I. M. *Am. J. Med. Genet.* **13**, 389–399 (1982).
3. Hook, E. B. & Warburton, D. *Hum. Genet.* **64**, 24–27 (1983).
4. Fernandez, R., Mendez, J. & Pasaro, E. *Hum. Genet.* **98**, 29–35 (1996).
5. Held, K. R. *et al.* *Hum. Genet.* **88**, 288–294 (1992).
6. Hsu, L. Y. *Am. J. Med. Genet.* **53**, 108–140 (1994).
7. Petrusevska, R. *et al.* *Clin. Genet.* **49**, 261–266 (1996).
8. Vogel, S. A. *J. Learn. Disabil.* **23**, 44–52 (1990).
9. Jacobs, P. A. *Science* **189**, 1044–1045 (1975).

Skuse and Jacobs reply — Henn and Zang argue that it is the presence of Y-chromosome sequences (presumably acting independently of sex steroids), rather than an imprinted gene on the X, that accounts both for male vulnerability to neurodevelopmental disorders of social cognition¹ and for the poorer social adjustment of girls who have Turner's syndrome, with 45, X^M rather than 45, X^P . Unfortunately, without direct observations on the chromosome constitution of brain cells, Henn and Zang's hypothesis is not testable.

The weight of evidence is against a large number of cryptic Y chromosome mosaics among the 45, X^M girls with Turner's syndrome. We initially examined 100 cells from peripheral blood of 211 girls with Turner's syndrome and found only 12 to have Y-chromosome sequences; all were

mosaics with a 45,X cell line and a cell line containing a structurally abnormal Y.

We subsequently tested for cryptic mosaicism using four microsatellites situated along the length of the Y chromosome, used at a sensitivity of 1 in 1,000 or greater, the tests being done without knowledge of the cytogenetic findings. Only the 12 patients known to contain Y chromosomes were positive. If cryptic mosaicism involving the Y chromosome is the explanation for our observations^{2,3}, it would have to be remarkably common, as 38% of 45, X^M females had higher social-dysfunction scores than any in the 45, X^P group. Cryptic mosaicism would also have to be remarkably localized, as we found no evidence of Y-bearing cells in the blood, although we accept that mosaicism can be unevenly distributed between tissues. We found no evidence of masculinization of 45, X^M females in any other respect.

Henn and Zang are correct to point out that there is greater variability of cognitive function within the X^M than in the X^P subjects. We considered the possibility that this might reflect two populations, one who had lost a Y chromosome and one who had lost an X. However, there is no way at present of differentiating between these groups and the idea seemed implausible.

We agree with the final point about the potential interest of XYY males as well as Klinefelter's syndrome (XXY). In about half of XXY cases the extra X is maternally derived and in the other half it is paternally derived. This is a subject we wish to study further in the near future.

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1. Baron-Cohen, S. & Hammer, J. *Adv. Infancy Res.* **11**, 193–217 (1997).
2. Skuse, D. H. *et al.* *Nature* **387**, 705–708 (1997).
3. Jacobs, P. A. *et al.* *Ann. Hum. Genet.* (in the press).

Detoxifying aluminium with buckwheat

Aluminium toxicity is a major problem limiting crop production in acid soils, which account for around 40% of the world's arable land¹. Some plants have developed strategies to avoid or tolerate aluminium, including buckwheat (*Fagopyrum esculentum* Moench cv. Jianxi), which has a high resistance to aluminium, but the mechanism responsible for resistance is not known. We have found that the aluminium-resistant Juanxi cultivar of buckwheat secretes oxalic acid from its roots specifically and quickly in response to aluminium stress. Further, aluminium accu-

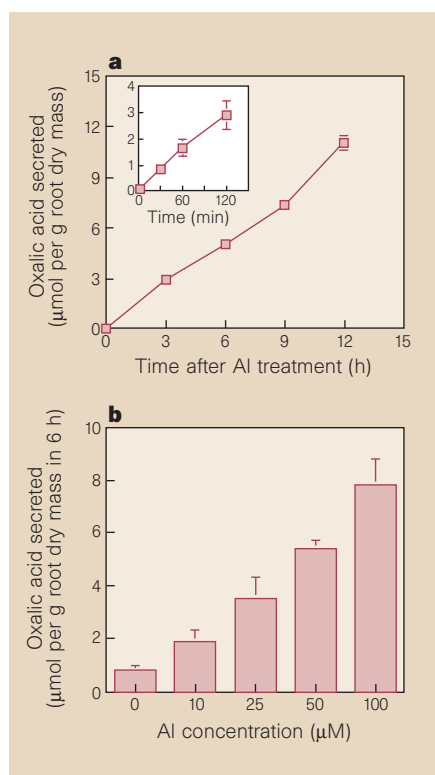


Figure 1 Oxalic acid secretion by buckwheat roots (*Fagopyrum esculentum* Moench cv. Jianxi) in response to aluminium stress. **a**, Time course of oxalic acid secretion after treatment with 50 μM AlCl₃ in 0.5 mM CaCl₂ solution at pH 4.5. **b**, Effect of aluminium concentration on the secretion of oxalic acid. Oxalic acid was detected by high-performance liquid chromatography. Plotted values are means ± s.e.m. of three replicates.

mulates in the leaf cells in a non-toxic Al-oxalate complex with a 1:3 ratio of aluminium to oxalic acid.

When we exposed buckwheat roots to aluminium chloride solution, oxalic acid was secreted within 30 minutes. The amount secreted increased linearly with treatment time (Fig. 1a) and with the external aluminium concentration (Fig. 1b). Neither phosphorus deficiency nor toxic metals such as lanthanum could induce the secretion of oxalic acid, indicating that this response is specific to aluminium stress.

The aluminium concentration in the buckwheat leaves reached about 450 mg per kg dry mass after aluminium treatment, in contrast to other species such as wheat and rape, which accumulated less than 50 mg per kg. The concentration of aluminium in extracted cell sap was as high as 2.03 mM, which accounts for about 90% of the total aluminium in the leaves.

We investigated the form of aluminium in the leaves using ²⁷Al nuclear magnetic resonance (NMR). We found only one signal at a chemical shift of 16.9 p.p.m. in the intact leaves and at 16.1 p.p.m. in the

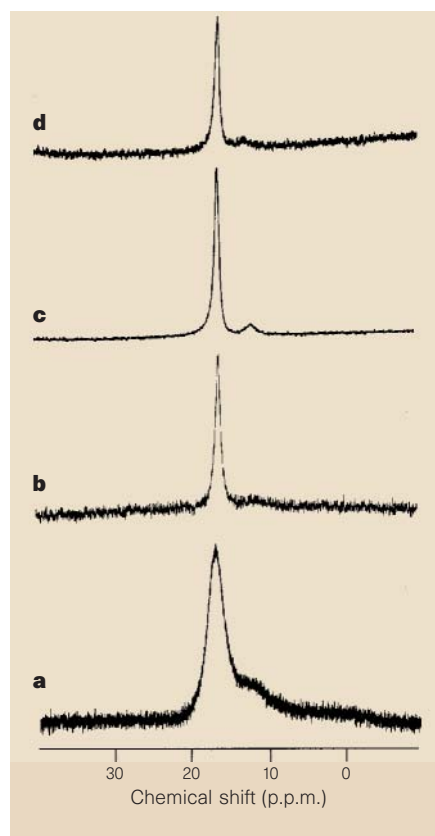


Figure 2 ²⁷Al-NMR spectra. **a**, Intact buckwheat leaves exposed to AlCl₃ solution. **b**, Crude cell sap from the leaves. **c**, Purified sap. **d**, 1:3 Al-oxalate complex. The aluminium complex in the sap was purified by Sephadex G-10. Spectra were obtained at 156.3 MHz using a JNM-α600 spectrometer.

crude cell sap (Fig. 2a, b), suggesting that the aluminium form in both samples is in a hexacoordinated complex². The chemical shift suggested that the aluminium is chelated with organic acids so we analysed organic acids in the cell sap. The main organic acid is oxalic acid, present at a concentration of about 50 mM in the cell sap. As oxalic acid can form a complex with aluminium at an aluminium:oxalic acid molar ratio of 1:1, 1:2 or 1:3, we compared the chemical shifts for these three complexes with the cell sap at the same pH (pH 4.6). The chemical shift of the 1:3 Al-oxalate complex is consistent with that in the leaves (Fig. 2d).

We purified the cell sap by molecular sieve chromatography. After four rounds of purification, the ratio of oxalic acid to aluminium decreased from about 25 to nearly 3. The chemical shift of ²⁷Al in the purified cell sap was consistent with those of intact leaves and crude cell sap (Fig. 2c). Further, the chemical shift of ¹³C in purified cell sap is consistent with that of the Al-oxalate (1:3) complex on a ¹³C-NMR spectrum.

The primary response of plants to aluminium toxicity is the inhibition of root

elongation^{3–5}. We examined the effects of the purified cell sap on the root elongation of corn. A 20-hour treatment with 20 μM AlCl₃ in 0.1 mM CaCl₂ solution inhibited root elongation of corn by 50%, whereas the purified cell sap containing the same concentration of aluminium did not inhibit root elongation, indicating that the 1:3 Al-oxalate complex is not phytotoxic. We stained the roots of wheat (cv. Scout 66) using eriochrome cyanine R. We found that the root apex, the critical site for elongation, treated with Al³⁺ became heavily stained, but roots treated with 1:2 and 1:3 Al-oxalate complexes were not stained, suggesting that oxalic acid prevents binding of aluminium to the cellular components, thereby detoxifying aluminium.

Several mechanisms for detoxifying metals have been reported. Metallothioneins and phytochelatins have been suggested to be involved in the detoxification of cadmium and copper⁶. Free histidine has been reported to be coordinated with nickel in the nickel hyperaccumulation phenotype⁷. To our knowledge, this is the first report that oxalic acid, the simplest dicarboxylic acid, takes part in detoxifying aluminium in both external and internal processes.

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1. Foy, C. D., Chaney, R. L. & White, M. C. A. *Rev. Plant Physiol.* **29**, 511–566 (1978).
2. Haraguchi, H. & Fujiwara, S. *J. Phys. Chem.* **73**, 3467–3473 (1969).
3. Bennet, R. J., Breen, C. M. & Fey, M. V. *Env. Exp. Bot.* **27**, 91–104 (1987).
4. Ryan, P. R., DiTomaso, J. M. & Kochian, L. V. *J. Exp. Bot.* **44**, 437–446 (1993).
5. Kochian, L. V. A. *Rev. Plant Physiol. Plant Mol. Biol.* **46**, 237–260 (1995).
6. Steffens, J. C. A. *Rev. Plant Physiol. Plant Mol. Biol.* **41**, 553–575 (1990).
7. Kramer, U., Cotter-Howells, J. D., Charnock, J. M., Baker, A. J. M. & Smith, J. A. C. *Nature* **379**, 635–638 (1996).

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In for a penny, off for a pound

Venus Envy: A History of Cosmetic Surgery

by Elizabeth Haiken

Johns Hopkins University Press: 1997.

Pp. 354. \$24.95, £20.50

Richard Davenport-Hines

Small penises, flat breasts, unshapely noses and ageing faces are not unusual or life-threatening. Yet in the United States, during the twentieth century, they ceased to be regarded as an inevitable feature of human experience and have been redefined as medical problems deserving treatment, and more recently as pathologies or deformities requiring medical solutions.

Elizabeth Haiken, an assistant professor at the University of Tennessee, has made a meticulous study of this historical process. Horrible injuries sustained by troops in the First World War required new surgical techniques for repairing faces so that men could resume normal lives and employment. It was swiftly recognized that these advances had potential civilian applications too.

In 1921 (also the year of the first Miss America beauty pageant) several physicians met at the Chicago Athletic Club to discuss the formation of the American Association of Plastic Surgeons (AAPS). Haiken traces the development of the AAPS as part of the national trend towards plastic surgeons joining the medical profession and becoming a hierarchical organization of medical specialists. Issues of publicity and profit proved contentious in the embryonic sphere of plastic surgery and not until the 1940s was academic accreditation standardized.

Several characteristics contributed to Americans' enthusiasm for cosmetic surgery. The puritan heritage, with its anxious self-scourings of conscience and sometimes morbidly introspective self-criticism, produced a nation that took its personal miseries seriously. The attainment of human perfection is another ingrained American delusion. These two mentalities encouraged many Americans to define self-improvement as encompassing physical perfection achieved by surgery.

Haiken shows that the process of turning plastic surgery into medicine in the United States, whereby medical vocabulary and ideology came to dominate explanations of human behaviour, was decisive in this process. Plastic surgeons ratified the twentieth-century American tendency to pathologize the human condition.

The American fascination with psychological complexes, notably the inferiority complex hypothesized by Alfred Adler, provided compelling justification for cosmetic



Smell of success? Before and after a nose-job.

surgery to improve self-esteem. The transformation of 'beauty surgery' from quackery into a laudable remedy for ugliness and low self-esteem enforced the trend towards normative standards of external appearance, encapsulated by a phrase in the musical *A Chorus Line*, which Haiken remembers hearing as a child: "different is nice, but it sure ain't pretty; pretty is what it's about".

The decision to alter one's face or body surgically was received not as narcissism but pragmatism. It became another of the freedoms exemplified by the American way, in this case the freedom to re-invent oneself. Cosmetic surgery was accepted as a weapon in the American armoury of self-improvement and career betterment. "It's not just a matter of good-looking people going to work in Hollywood and bad-looking people digging ditches," an economic researcher reported in 1933. "Within any given occupation, good-looking people make more [money]."

Physical appearance became commodified. Haiken sees cosmetic surgery as the great forerunner of treatments such as human growth hormone and Prozac in which physicians respond to market demands by selling a new range of optional medical treatments. The promotion of face-lifts for women after 1945, and later of silicone breast implants,

with their controversial sequelae and side-effects, are case studies analysed by Haiken.

She also demonstrates the increasing diversity of cosmetic-surgery patients. Most early American plastic-surgery patients were women rather than war-wounded men. The outlaw John Dillinger attempted to disguise himself in 1935 with a nose-job and face-lift, but until the 1960s plastic surgeons regarded male interest in face-lifts as indicating homosexuality. But with increasing job uncertainty in the 1960s, men resorted to cosmetic surgery, and good looks "may represent nothing less than survival itself to a psychologically murdered salesman". The case of the pop singer Michael Jackson raises other questions of race and ethnicity: American patients have variously wanted to look less black, less Jewish, less Italian, or more Caucasian.

Haiken has written a humane, balanced history of cosmetic surgery, drawing with sensitivity and deftness on impressive archival sources, including surgeons' folders on prospective patients. Although she has reservations about its cultural imperatives, she is sympathetic and generous to its patients, and to most surgeons: she lets the chauvinistic and calculating extracts from Dr Robert Andrew Franklyn's 1960 book *Beauty Surgeon* condemn themselves.

Unlike some commentators, she does not treat recipients of cosmetic surgery as dupes, but places their anxieties and desires in a fascinating historical continuum. Her book is a first-class exercise in medical history, raising intriguing questions about normalization, ideological manipulation, gender, ethnicity and the profit motive in medicine.

Haiken also throws up many side issues. In 1935 the president of the AAPS was described as living in a luxurious penthouse equipped with two butlers: "at any moment the Doctor may have his whittled asparagus, hamburgers or sausages, toast, strawberry jam," reported a journalist. Fewer Americans might need liposuction, one reflects, if they did not have such foolish eating habits. □

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Elementary history

Traces of the Past: Unraveling the Secrets of Archaeology through Chemistry

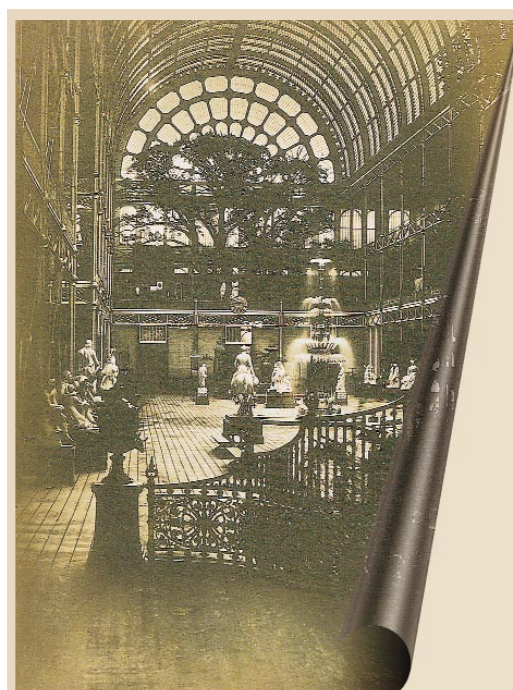
by Joseph B. Lambert
Addison-Wesley: 1997. Pp. 319. \$30

Robert Hedges

There is a long tradition of chemists applying their knowledge and skills to studying the remains of antiquity. Sir Humphry Davy, for example, attempted to discover the composition of a newly discovered synthetic pigment, now called Egyptian blue, which had been excavated from Pompeii in 1814. Joseph B. Lambert has also made a distinguished contribution to this tradition, but in *Traces of the Past* he remains modest about it.

Lambert provides thumbnail sketches of many pleasing applications of chemistry. Their impact on our knowledge of antiquity ranges from despatching minor curiosities to realigning archaeological foundations. The interested outsider will be surprised to see how extensive and sophisticated are the modern chemical techniques applied to archaeological questions. They include carbon-13 nuclear magnetic resonance, mass spectrometry in most of its acronyms (SIMS, GC/MS, AMS, IRMS, ICP-MS), capillary electrophoresis, and the polymerase chain reaction.

Many of the questions posed relate to artefacts, including humble domestic discards and items of exceptional workmanship and display. How were they made? How did they get to be where they were found? It is often possible, for example, to show that pots, as well as valuable items such as fine marble or metal ingots, were transported surprisingly large distances. These results allow us to build up a picture of trading relations. Other questions involve chemical



The dawn of photography

London's Victoria and Albert Museum has one of the oldest and finest collections of photographs in the world. Mark Haworth-Booth, the museum's curator of photographs, has chosen a selection of these images to illustrate his book *Photography: An Independent Art — Photographs from the Victoria and Albert Museum 1839–1996* (Princeton University Press, \$39.50; V&A Publications, £30). As well as describing the collection, the book gives a general history of photography. These pictures were taken at the Great Exhibition held in the Crystal Palace in 1851. Henry Cole was a driving force behind the exhibition and went on to found the museum and to take a keen interest in photography as an art form.



processes, such as why the outlined stains of a skeleton are all that remains after burial in certain soils. This also raises the important but notoriously difficult subject of dating materials from their degree of alteration after deposition.

In *Traces of the Past*, Lambert opens a window to a kaleidoscopic view of chemical applications. The book is organized by taking types of material in turn, with a sense of moving to the more artificial as one proceeds through it (from stones to pottery glazes, and so on).

The book does not make for easy continuous reading, as it is too concentrated and lacks overall themes of interest and excitement. This is a shame, because there are many charming vignettes. For example, the story of the technical sophistication required to produce Attic red-figured ware (the classic Greek vases whose masterly drawings show so much of life in ancient Athens) is fascinating; it gets even better when linked to the

chemistry of related black pigments, some based on manganese spinels.

I also enjoyed the etymological asides: 'miniature' comes from writing with the cinnabar pigment minium; the Swedish town of Ytterby is the source for nomination of no less than four chemical elements; and porcelain has a whiteness recalling a type of shell known as little pigs.

My ambivalence is perhaps due to my not being clear whom this book is aimed at. Much of the chemistry is explained, albeit exceedingly briefly, but a non-chemist would need great dedication to take in half the book. On the other hand, a chemist might look for more on chemical synthesis and structure. That said, the book is up to date and comprehensive, and gives examples of almost every type of application. □

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Insects bite back

Nature Wars: People vs. Pests

by Mark L. Winston

Harvard University Press: 1997. Pp. 210.

\$24.95, £16.50

Lawrence M. Hanks

Insects preceded humans on Earth by more than 300 million years, diversifying to occupy millions of ecological niches from fern-frond chewer to dinosaur-blood sucker. When finally our ancestors emerged from the African forest to conquer the savannah, they undoubtedly strode on legs that were flea-bitten and mosquito-welted. Who is to say that primate bipedalism did not evolve to free the hands for swatting bugs and scratching itches?

Ever since that time we have waged war against an insect world that consumes and contaminates our food plants in field and storage bin, pesters and weakens our domestic animals, and transmits our deadliest diseases. The technology of this war has advanced from the simple but effective step-and-squash to helicopter broadcast-spraying of synthetic insecticides and the manipulation of crop genomes to improve resistance to insect feeding.

Each technological innovation in pest control has promised final dominion over our flea-brained antagonists, but we have been outwitted at every turn. Insects have gracefully sidestepped our impressive arsenals of insecticides by evolving resistance to them, adapting to new formulations within a few years of their introduction. Although people have made huge strides in conquering the environment, replacing entire plant communities with agroecosystems and paving the earth to aid transportation, we have gained little ground in our battle with the insects. They continue to destroy much of our crops, invade our homes and businesses with impunity, and spread our diseases.

Nature Wars is an effective primer for the general reader on our struggles against insects and other pests. Mark L. Winston recounts how human activities have enticed innocuous animal species from their natural habitats to adapt and exploit our abundant resources, and how we have failed miserably in mastering these 'pests'. Our foibles are beautifully, and painfully, illustrated by the control programmes that have been devised for the forest-defoliating gypsy moth, the apple-infesting codling moth, and the disgust-inducing cockroach.

In an articulate and accessible writing style, Winston explains the pesticide dilemma, the threat that our reliance on synthetic pesticides poses both to human health and safety and to the preservation of what is left of the natural environment. He describes alter-

native approaches to pest management that might resolve this dilemma, such as the use of insect sex pheromones and biological controls, such as insect parasites, predators and pathogens. The development and implementation of these alternative tactics are currently stymied by economics, the bureaucracy that regulates pest-management practices, and the general hypersensitivity of people to insects.

Our progress in escaping the pesticide dilemma depends on developing effective and socially acceptable alternatives to pest management, yet some of these alternatives have recently come under attack. Ironically, it is environmental groups that have emphatically protested against the use of microbial insecticides and transgenic (biologically engineered) plants in food production. Winston's discussion of these controversial issues, and the conflict between humans and pests, will be helpful to anyone who hopes to develop an informed opinion about our continuing war with nature. □

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Reliable recipes

Correcting the Blueprint of Life: An Historical Account of the Discovery of DNA Repair Mechanisms

by Errol C. Friedberg

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A consequence of living surrounded by water, oxygen, radiation and reactive chemicals is that the genes in every organism continually decay and are bombarded by destructive environmental agents. Cells survive because they have a battery of defensive mechanisms to repair such DNA damage.

During the period covered by Errol C. Friedberg's account, the 1940s to the mid-1970s, the study of DNA repair was outside mainstream molecular biology. It is now recognized as a discipline of great importance with an influence on many areas of biology, and it is timely to look back

Plants on the catwalk



The herbarium of the Natural History Museum in London is one of the largest in the world, containing six million dried and pressed plants. But it is much more than a mortuary for famous collections of dead plants or a scientific resource for taxonomists: many of the specimens are beautiful objects in their own right. In *Flora* (Schirmer/Mosel, Munich; £40),

the fashion photographer Nick Knight has turned his lens to capture the "fragile corporeality, filigree transparency and stunning colours" of 46 specimens (shown here are *Stapelia gettiffiei* (left) and *Clidemia octona* (right)), while the botanist Sandra Knapp weaves a compelling story — be it cultural, historical or scientific — around each plant.

on its pioneering days.

Richard Dawkins has pointed out that the metaphor of a blueprint is inadequate for DNA in almost every particular, as the genome is not a homunculus but a code of instructions more akin to a recipe. The image of recipe correction is admittedly less than compelling. Thankfully Friedberg provides real inspiration by recounting some of the rich experiences that the scientific life can offer. We are treated to descriptions of sudden flashes of insight over a dram of whisky at a conference in the Scottish highlands, pivotal blackboard discussions with students, and colleagues taking Sunday afternoon walks while planning experiments and searching for fossils.

Friedberg has turned up some valuable fossils himself, particularly in the form of the original letters and diaries of Albert Kellner. These are essential sources for a narrative on the discovery of enzymatic photoreactivation, in which damage induced by ultraviolet radiation is repaired by a protein that channels energy from visible light to DNA and reverses the injury. This engaging story peaks with a happily resolved priority dispute.

The discoveries of nucleotide and base excision repair, mismatch repair and direct reversal of DNA methylation by a self-inactivating enzyme are treated in turn. The unorthodox discovery of the 'SOS response' in the bacterium *Escherichia coli*

is covered in some detail, as it not only gives insights into the error-prone bypass of damage by DNA polymerases but also provided the first evidence for a biological system in which the expression of a large group of genes is controlled by a single repressor.

In several cases, investigators delayed publication of their work to give colleagues a chance to catch up, or even refused to publish similar data because they had not done enough experiments. This seems a world away from some of today's attitudes, where arguments about which author is listed last on a collaborative paper can deteriorate into an exchange of apoplectic e-mails. But there are also examples of unresolved priority battles, years in the cold room with no results, and instances where new (and correct) ideas were ignored by more established senior figures.

(Tense episodes have always been a feature of the creative arts and sciences. Take the story of a young Franz Liszt encountering a very old man in Leipzig who had been a chorister during the last days of J. S. Bach. Delighted by the chance to learn about authentic performance practice during a musical golden age, Liszt queried the man, only to be told: "It always went wretchedly. Then we were beaten".)

While describing the discovery of DNA glycosylases, Friedberg uses the occasion for some self-flagellation over missed opportunities. This precipitates the only

explanatory figure in the book, showing the mechanism of pyrimidine dimer removal from DNA as initiated by the unusual dimer-nicking enzymes of bacteriophage T4 and *Micrococcus luteus*. But the accompanying text is rather confusing. Superb visual material is provided however in a collection of photographs of some of the principals in the story (including the famous one of Luria without his shirt on).

For those currently investigating DNA repair, this fluent account is essential reading, and would also be a fine complement to courses on the history of molecular biology. To the uninitiated, some of the concepts will be difficult to follow without further diagrams and a basic knowledge of molecular biology.

Friedberg writes from his perspective as a participant in some of the unfolding events and a friend of many of those involved, so the exposition is inevitably coloured. But he has made an invaluable contribution by documenting primary sources and setting the stage for further study of the history of this field. □

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Experimental quantum teleportation

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Quantum teleportation—the transmission and reconstruction over arbitrary distances of the state of a quantum system—is demonstrated experimentally. During teleportation, an initial photon which carries the polarization that is to be transferred and one of a pair of entangled photons are subjected to a measurement such that the second photon of the entangled pair acquires the polarization of the initial photon. This latter photon can be arbitrarily far away from the initial one. Quantum teleportation will be a critical ingredient for quantum computation networks.

The dream of teleportation is to be able to travel by simply reappearing at some distant location. An object to be teleported can be fully characterized by its properties, which in classical physics can be determined by measurement. To make a copy of that object at a distant location one does not need the original parts and pieces—all that is needed is to send the scanned information so that it can be used for reconstructing the object. But how precisely can this be a true copy of the original? What if these parts and pieces are electrons, atoms and molecules? What happens to their individual quantum properties, which according to the Heisenberg's uncertainty principle cannot be measured with arbitrary precision?

Bennett *et al.*¹ have suggested that it is possible to transfer the quantum state of a particle onto another particle—the process of quantum teleportation—provided one does not get any information about the state in the course of this transformation. This requirement can be fulfilled by using entanglement, the essential feature of quantum mechanics². It describes correlations between quantum systems much stronger than any classical correlation could be.

The possibility of transferring quantum information is one of the cornerstones of the emerging field of quantum communication and quantum computation³. Although there is fast progress in the theoretical description of quantum information processing, the difficulties in handling quantum systems have not allowed an equal advance in the experimental realization of the new proposals. Besides the promising developments of quantum cryptography⁴ (the first provably secure way to send secret messages), we have only recently succeeded in demonstrating the possibility of quantum dense coding⁵, a way to quantum mechanically enhance data compression. The main reason for this slow experimental progress is that, although there exist methods to produce pairs of entangled photons⁶, entanglement has been demonstrated for atoms only very recently⁷ and it has not been possible thus far to produce entangled states of more than two quanta.

Here we report the first experimental verification of quantum teleportation. By producing pairs of entangled photons by the process of parametric down-conversion and using two-photon interferometry for analysing entanglement, we could transfer a quantum property (in our case the polarization state) from one photon to another. The methods developed for this experiment will be of great importance both for exploring the field of quantum communication and for future experiments on the foundations of quantum mechanics.

The problem

To make the problem of transferring quantum information clearer, suppose that Alice has some particle in a certain quantum state $|\psi\rangle$

and she wants Bob, at a distant location, to have a particle in that state. There is certainly the possibility of sending Bob the particle directly. But suppose that the communication channel between Alice and Bob is not good enough to preserve the necessary quantum coherence or suppose that this would take too much time, which could easily be the case if $|\psi\rangle$ is the state of a more complicated or massive object. Then, what strategy can Alice and Bob pursue?

As mentioned above, no measurement that Alice can perform on $|\psi\rangle$ will be sufficient for Bob to reconstruct the state because the state of a quantum system cannot be fully determined by measurements. Quantum systems are so evasive because they can be in a superposition of several states at the same time. A measurement on the quantum system will force it into only one of these states—this is often referred to as the projection postulate. We can illustrate this important quantum feature by taking a single photon, which can be horizontally or vertically polarized, indicated by the states $|\leftrightarrow\rangle$ and $|\updownarrow\rangle$. It can even be polarized in the general superposition of these two states

$$|\psi\rangle = \alpha|\leftrightarrow\rangle + \beta|\updownarrow\rangle \quad (1)$$

where α and β are two complex numbers satisfying $|\alpha|^2 + |\beta|^2 = 1$. To place this example in a more general setting we can replace the states $|\leftrightarrow\rangle$ and $|\updownarrow\rangle$ in equation (1) by $|0\rangle$ and $|1\rangle$, which refer to the states of any two-state quantum system. Superpositions of $|0\rangle$ and $|1\rangle$ are called qubits to signify the new possibilities introduced by quantum physics into information science⁸.

If a photon in state $|\psi\rangle$ passes through a polarizing beamsplitter—a device that reflects (transmits) horizontally (vertically) polarized photons—it will be found in the reflected (transmitted) beam with probability $|\alpha|^2$ ($|\beta|^2$). Then the general state $|\psi\rangle$ has been projected either onto $|\leftrightarrow\rangle$ or onto $|\updownarrow\rangle$ by the action of the measurement. We conclude that the rules of quantum mechanics, in particular the projection postulate, make it impossible for Alice to perform a measurement on $|\psi\rangle$ by which she would obtain all the information necessary to reconstruct the state.

The concept of quantum teleportation

Although the projection postulate in quantum mechanics seems to bring Alice's attempts to provide Bob with the state $|\psi\rangle$ to a halt, it was realised by Bennett *et al.*¹ that precisely this projection postulate enables teleportation of $|\psi\rangle$ from Alice to Bob. During teleportation Alice will destroy the quantum state at hand while Bob receives the quantum state, with neither Alice nor Bob obtaining information about the state $|\psi\rangle$. A key role in the teleportation scheme is played by an entangled ancillary pair of particles which will be initially shared by Alice and Bob.

Suppose particle 1 which Alice wants to teleport is in the initial state $|\psi\rangle_1 = \alpha|\leftrightarrow\rangle_1 + \beta|\uparrow\rangle_1$ (Fig. 1a), and the entangled pair of particles 2 and 3 shared by Alice and Bob is in the state:

$$|\psi^- \rangle_{23} = \frac{1}{\sqrt{2}} (|\leftrightarrow\rangle_2 |\uparrow\rangle_3 - |\uparrow\rangle_2 |\leftrightarrow\rangle_3) \quad (2)$$

That entangled pair is a single quantum system in an equal superposition of the states $|\leftrightarrow\rangle_2 |\uparrow\rangle_3$ and $|\uparrow\rangle_2 |\leftrightarrow\rangle_3$. The entangled state contains no information on the individual particles; it only indicates that the two particles will be in opposite states. The important property of an entangled pair is that as soon as a measurement on one of the particles projects it, say, onto $|\leftrightarrow\rangle$ the state of the other one is determined to be $|\uparrow\rangle$, and vice versa. How could a measurement on one of the particles instantaneously influence the state of the other particle, which can be arbitrarily

far away? Einstein, among many other distinguished physicists, could simply not accept this “spooky action at a distance”. But this property of entangled states has now been demonstrated by numerous experiments (for reviews, see refs 9, 10).

The teleportation scheme works as follows. Alice has the particle 1 in the initial state $|\psi\rangle_1$ and particle 2. Particle 2 is entangled with particle 3 in the hands of Bob. The essential point is to perform a specific measurement on particles 1 and 2 which projects them onto the entangled state:

$$|\psi^- \rangle_{12} = \frac{1}{\sqrt{2}} (|\leftrightarrow\rangle_1 |\uparrow\rangle_2 - |\uparrow\rangle_1 |\leftrightarrow\rangle_2) \quad (3)$$

This is only one of four possible maximally entangled states into which any state of two particles can be decomposed. The projection of an arbitrary state of two particles onto the basis of the four states is called a Bell-state measurement. The state given in equation (3) distinguishes itself from the three other maximally entangled states by the fact that it changes sign upon interchanging particle 1 and particle 2. This unique antisymmetric feature of $|\psi^- \rangle_{12}$ will play an important role in the experimental identification, that is, in measurements of this state.

Quantum physics predicts¹ that once particles 1 and 2 are projected into $|\psi^- \rangle_{12}$, particle 3 is instantaneously projected into the initial state of particle 1. The reason for this is as follows. Because we observe particles 1 and 2 in the state $|\psi^- \rangle_{12}$ we know that whatever the state of particle 1 is, particle 2 must be in the opposite state, that is, in the state orthogonal to the state of particle 1. But we had initially prepared particle 2 and 3 in the state $|\psi^- \rangle_{23}$, which means that particle 2 is also orthogonal to particle 3. This is only possible if particle 3 is in the same state as particle 1 was initially. The final state of particle 3 is therefore:

$$|\psi\rangle_3 = \alpha|\leftrightarrow\rangle_3 + \beta|\uparrow\rangle_3 \quad (4)$$

We note that during the Bell-state measurement particle 1 loses its identity because it becomes entangled with particle 2. Therefore the state $|\psi\rangle_1$ is destroyed on Alice’s side during teleportation.

This result (equation (4)) deserves some further comments. The transfer of quantum information from particle 1 to particle 3 can happen over arbitrary distances, hence the name teleportation. Experimentally, quantum entanglement has been shown¹¹ to survive over distances of the order of 10 km. We note that in the teleportation scheme it is not necessary for Alice to know where Bob is. Furthermore, the initial state of particle 1 can be completely unknown not only to Alice but to anyone. It could even be quantum mechanically completely undefined at the time the Bell-state measurement takes place. This is the case when, as already remarked by Bennett *et al.*¹, particle 1 itself is a member of an entangled pair and therefore has no well-defined properties on its own. This ultimately leads to entanglement swapping^{12,13}.

It is also important to notice that the Bell-state measurement does not reveal any information on the properties of any of the particles. This is the very reason why quantum teleportation using coherent two-particle superpositions works, while any measurement on one-particle superpositions would fail. The fact that no information whatsoever is gained on either particle is also the reason why quantum teleportation escapes the verdict of the no-cloning theorem¹⁴. After successful teleportation particle 1 is not available in its original state any more, and therefore particle 3 is not a clone but is really the result of teleportation.

A complete Bell-state measurement can not only give the result that the two particles 1 and 2 are in the antisymmetric state, but with equal probabilities of 25% we could find them in any one of the three other entangled states. When this happens, particle 3 is left in one of three different states. It can then be brought by Bob into the original state of particle 1 by an accordingly chosen transformation, independent of the state of particle 1, after receiving via a classical communication channel the information on which of the Bell-state

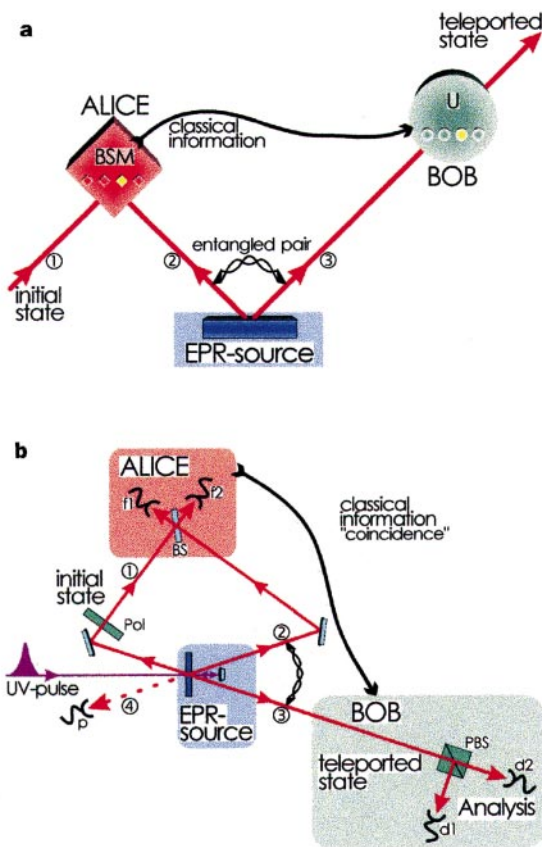


Figure 1 Scheme showing principles involved in quantum teleportation (a) and the experimental set-up (b). **a**, Alice has a quantum system, particle 1, in an initial state which she wants to teleport to Bob. Alice and Bob also share an ancillary entangled pair of particles 2 and 3 emitted by an Einstein–Podolsky–Rosen (EPR) source. Alice then performs a joint Bell-state measurement (BSM) on the initial particle and one of the ancillaries, projecting them also onto an entangled state. After she has sent the result of her measurement as classical information to Bob, he can perform a unitary transformation (U) on the other ancillary particle resulting in it being in the state of the original particle. **b**, A pulse of ultraviolet radiation passing through a nonlinear crystal creates the ancillary pair of photons 2 and 3. After retroreflection during its second passage through the crystal the ultraviolet pulse creates another pair of photons, one of which will be prepared in the initial state of photon 1 to be teleported, the other one serving as a trigger indicating that a photon to be teleported is under way. Alice then looks for coincidences after a beam splitter BS where the initial photon and one of the ancillaries are superposed. Bob, after receiving the classical information that Alice obtained a coincidence count in detectors f1 and f2 identifying the $|\psi^- \rangle_{12}$ Bell state, knows that his photon 3 is in the initial state of photon 1 which he then can check using polarization analysis with the polarizing beam splitter PBS and the detectors d1 and d2. The detector p provides the information that photon 1 is under way.

results was obtained by Alice. Yet we note, with emphasis, that even if we chose to identify only one of the four Bell states as discussed above, teleportation is successfully achieved, albeit only in a quarter of the cases.

Experimental realization

Teleportation necessitates both production and measurement of entangled states; these are the two most challenging tasks for any experimental realization. Thus far there are only a few experimental techniques by which one can prepare entangled states, and there exist no experimentally realized procedures to identify all four Bell states for any kind of quantum system. However, entangled pairs of photons can readily be generated and they can be projected onto at least two of the four Bell states.

We produced the entangled photons 2 and 3 by parametric down-conversion. In this technique, inside a nonlinear crystal, an incoming pump photon can decay spontaneously into two photons which, in the case of type II parametric down-conversion, are in the state given by equation (2) (Fig. 2)⁶.

To achieve projection of photons 1 and 2 into a Bell state we have to make them indistinguishable. To achieve this indistinguishability we superpose the two photons at a beam splitter (Fig. 1b). Then if they are incident one from each side, how can it happen that they emerge still one on each side? Clearly this can happen if they are either both reflected or both transmitted. In quantum physics we have to superimpose the amplitudes for these two possibilities. Unitarity implies that the amplitude for both photons being reflected obtains an additional minus sign. Therefore, it seems that the two processes cancel each other. This is, however, only true for a symmetric input state. For an antisymmetric state, the two possibilities obtain another relative minus sign, and therefore they constructively interfere^{15,16}. It is thus sufficient for projecting photons 1 and 2 onto the antisymmetric state $|\psi\rangle_{12}$ to place detectors in each of the outputs of the beam splitter and to register simultaneous detections (coincidence)^{17–19}.

To make sure that photons 1 and 2 cannot be distinguished by their arrival times, they were generated using a pulsed pump beam and sent through narrow-bandwidth filters producing a coherence time much longer than the pump pulse length²⁰. In the experiment,

the pump pulses had a duration of 200 fs at a repetition rate of 76 MHz. Observing the down-converted photons at a wavelength of 788 nm and a bandwidth of 4 nm results in a coherence time of 520 fs. It should be mentioned that, because photon 1 is also produced as part of an entangled pair, its partner can serve to indicate that it was emitted.

How can one experimentally prove that an unknown quantum state can be teleported? First, one has to show that teleportation works for a (complete) basis, a set of known states into which any other state can be decomposed. A basis for polarization states has just two components, and in principle we could choose as the basis horizontal and vertical polarization as emitted by the source. Yet this would not demonstrate that teleportation works for any general superposition, because these two directions are preferred directions in our experiment. Therefore, in the first demonstration we choose as the basis for teleportation the two states linearly polarized at -45° and $+45^\circ$ which are already superpositions of the horizontal and vertical polarizations. Second, one has to show that teleportation works for superpositions of these base states. Therefore we also demonstrate teleportation for circular polarization.

Results

In the first experiment photon 1 is polarized at 45° . Teleportation should work as soon as photon 1 and 2 are detected in the $|\psi\rangle_{12}$ state, which occurs in 25% of all possible cases. The $|\psi\rangle_{12}$ state is identified by recording a coincidence between two detectors, f1 and f2, placed behind the beam splitter (Fig. 1b).

If we detect a f1f2 coincidence (between detectors f1 and f2), then photon 3 should also be polarized at 45° . The polarization of photon 3 is analysed by passing it through a polarizing beam splitter selecting $+45^\circ$ and -45° polarization. To demonstrate teleportation, only detector d2 at the $+45^\circ$ output of the polarizing beam splitter should click (that is, register a detection) once detectors f1 and f2 click. Detector d1 at the -45° output of the polarizing beam splitter should not detect a photon. Therefore, recording a three-fold coincidence d2f1f2 ($+45^\circ$ analysis) together with the absence of a three-fold coincidence d1f1f2 (-45° analysis) is a proof that the polarization of photon 1 has been teleported to photon 3.

To meet the condition of temporal overlap, we change in small

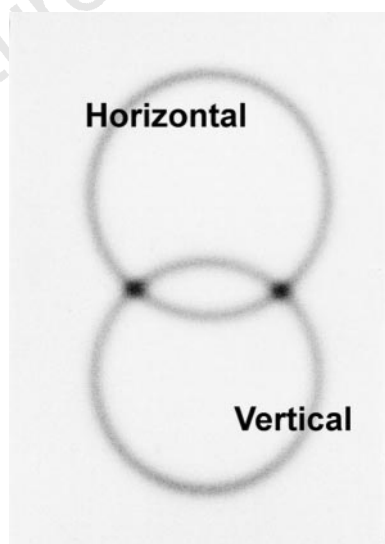


Figure 2 Photons emerging from type II down-conversion (see text). Photograph taken perpendicular to the propagation direction. Photons are produced in pairs. A photon on the top circle is horizontally polarized while its exactly opposite partner in the bottom circle is vertically polarized. At the intersection points their polarizations are undefined; all that is known is that they have to be different, which results in entanglement.

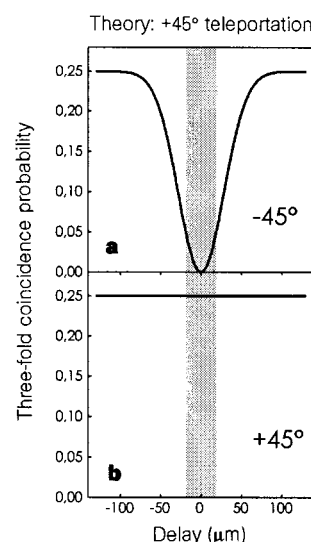


Figure 3 Theoretical prediction for the three-fold coincidence probability between the two Bell-state detectors (f1, f2) and one of the detectors analysing the teleported state. The signature of teleportation of a photon polarization state at $+45^\circ$ is a dip to zero at zero delay in the three-fold coincidence rate with the detector analysing -45° (d1f1f2) (a) and a constant value for the detector analysis $+45^\circ$ (d2f1f2) (b). The shaded area indicates the region of teleportation.

steps the arrival time of photon 2 by changing the delay between the first and second down-conversion by translating the retroreflection mirror (Fig. 1b). In this way we scan into the region of temporal overlap at the beam splitter so that teleportation should occur.

Outside the region of teleportation, photon 1 and 2 each will go either to f1 or to f2 independent of one another. The probability of having a coincidence between f1 and f2 is therefore 50%, which is twice as high as inside the region of teleportation. Photon 3 should not have a well-defined polarization because it is part of an entangled pair. Therefore, d1 and d2 have both a 50% chance of receiving photon 3. This simple argument yields a 25% probability both for the -45° analysis (d1f1f2 coincidences) and for the $+45^\circ$ analysis (d2f1f2 coincidences) outside the region of teleportation. Figure 3 summarizes the predictions as a function of the delay. Successful teleportation of the $+45^\circ$ polarization state is then characterized by a decrease to zero in the -45° analysis (Fig. 3a), and by a constant value for the $+45^\circ$ analysis (Fig. 3b).

The theoretical prediction of Fig. 3 may easily be understood by realizing that at zero delay there is a decrease to half in the coincidence rate for the two detectors of the Bell-state analyser, f1 and f2, compared with outside the region of teleportation. Therefore, if the polarization of photon 3 were completely uncorrelated to the others the three-fold coincidence should also show this dip to half. That the right state is teleported is indicated by the fact that the dip goes to zero in Fig. 3a and that it is filled to a flat curve in Fig. 3b.

We note that equally as likely as the production of photons 1, 2 and 3 is the emission of two pairs of down-converted photons by a single source. Although there is no photon coming from the first source (photon 1 is absent), there will still be a significant contribution to the three-fold coincidence rates. These coincidences have nothing to do with teleportation and can be identified by blocking the path of photon 1.

The probability for this process to yield spurious two- and three-fold coincidences can be estimated by taking into account the experimental parameters. The experimentally determined value

Table 1 Visibility of teleportation in three fold coincidences

Polarization	Visibility
$+45^\circ$	0.63 ± 0.02
-45°	0.64 ± 0.02
0°	0.66 ± 0.02
90°	0.61 ± 0.02
Circular	0.57 ± 0.02

for the percentage of spurious three-fold coincidences is $68\% \pm 1\%$. In the experimental graphs of Fig. 4 we have subtracted the experimentally determined spurious coincidences.

The experimental results for teleportation of photons polarized under $+45^\circ$ are shown in the left-hand column of Fig. 4; Fig. 4a and b should be compared with the theoretical predictions shown in Fig. 3. The strong decrease in the -45° analysis, and the constant signal for the $+45^\circ$ analysis, indicate that photon 3 is polarized along the direction of photon 1, confirming teleportation.

The results for photon 1 polarized at -45° demonstrate that teleportation works for a complete basis for polarization states (right-hand column of Fig. 4). To rule out any classical explanation for the experimental results, we have produced further confirmation that our procedure works by additional experiments. In these experiments we teleported photons linearly polarized at 0° and at 90° , and also teleported circularly polarized photons. The experimental results are summarized in Table 1, where we list the visibility of the dip in three-fold coincidences, which occurs for analysis orthogonal to the input polarization.

As mentioned above, the values for the visibilities are obtained after subtracting the offset caused by spurious three-fold coincidences. These can experimentally be excluded by conditioning the three-fold coincidences on the detection of photon 4, which effectively projects photon 1 into a single-particle state. We have performed this four-fold coincidence measurement for the case of teleportation of the $+45^\circ$ and $+90^\circ$ polarization states, that is, for two non-orthogonal

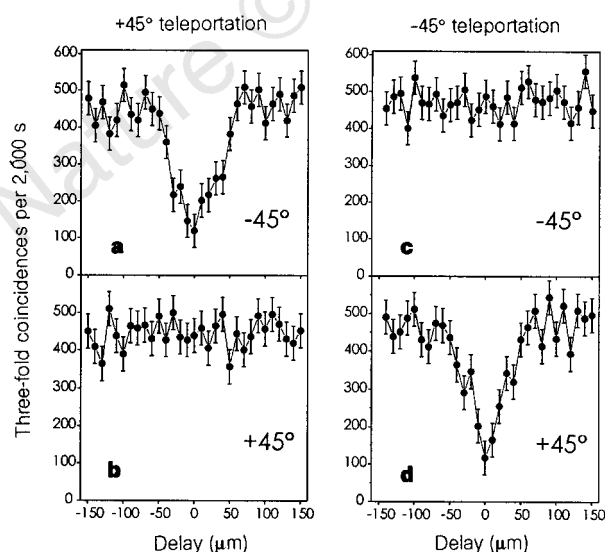


Figure 4 Experimental results. Measured three-fold coincidence rates d1f1f2 (-45°) and d2f1f2 ($+45^\circ$) in the case that the photon state to be teleported is polarized at $+45^\circ$ (a and b) or at -45° (c and d). The coincidence rates are plotted as function of the delay between the arrival of photon 1 and 2 at Alice's beam splitter (see Fig. 1b). The three-fold coincidence rates are plotted after subtracting the spurious three-fold contribution (see text). These data, compared with Fig. 3, together with similar ones for other polarizations (Table 1) confirm teleportation for an arbitrary state.

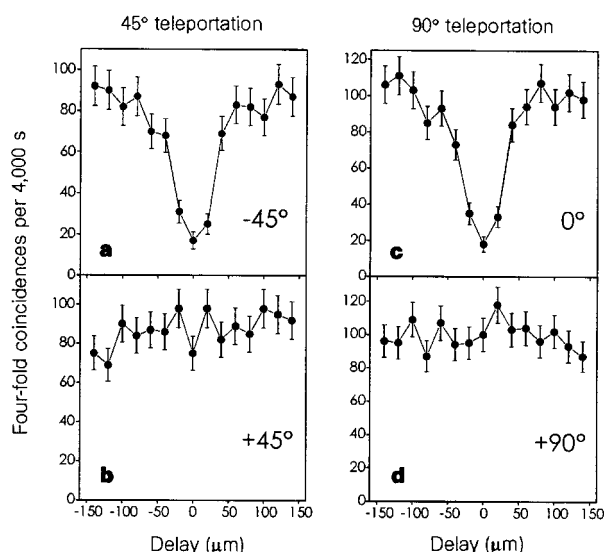


Figure 5 Four-fold coincidence rates (without background subtraction). Conditioning the three-fold coincidences as shown in Fig. 4 on the registration of photon 4 (see Fig. 1b) eliminates the spurious three-fold background. a and b show the four-fold coincidence measurements for the case of teleportation of the $+45^\circ$ polarization state; c and d show the results for the $+90^\circ$ polarization state. The visibilities, and thus the polarizations of the teleported photons, obtained without any background subtraction are $70\% \pm 3\%$. These results for teleportation of two non-orthogonal states prove that we have demonstrated teleportation of the quantum state of a single photon.

states. The experimental results are shown in Fig. 5. Visibilities of $70\% \pm 3\%$ are obtained for the dips in the orthogonal polarization states. Here, these visibilities are directly the degree of polarization of the teleported photon in the right state. This proves that we have demonstrated teleportation of the quantum state of a single photon.

The next steps

In our experiment, we used pairs of polarization entangled photons as produced by pulsed down-conversion and two-photon interferometric methods to transfer the polarization state of one photon onto another one. But teleportation is by no means restricted to this system. In addition to pairs of entangled photons or entangled atoms^{7,21}, one could imagine entangling photons with atoms, or phonons with ions, and so on. Then teleportation would allow us to transfer the state of, for example, fast-decohering, short-lived particles, onto some more stable systems. This opens the possibility of quantum memories, where the information of incoming photons is stored on trapped ions, carefully shielded from the environment.

Furthermore, by using entanglement purification²²—a scheme of improving the quality of entanglement if it was degraded by decoherence during storage or transmission of the particles over noisy channels—it becomes possible to teleport the quantum state of a particle to some place, even if the available quantum channels are of very poor quality and thus sending the particle itself would very probably destroy the fragile quantum state. The feasibility of preserving quantum states in a hostile environment will have great advantages in the realm of quantum computation. The teleportation scheme could also be used to provide links between quantum computers.

Quantum teleportation is not only an important ingredient in quantum information tasks; it also allows new types of experiments and investigations of the foundations of quantum mechanics. As any arbitrary state can be teleported, so can the fully undetermined state of a particle which is member of an entangled pair. Doing so, one transfers the entanglement between particles. This allows us not only to chain the transmission of quantum states over distances, where decoherence would have already destroyed the state completely, but it also enables us to perform a test of Bell's theorem on particles which do not share any common past, a new step in the investigation of the features of quantum mechanics. Last but not least, the discussion about the local realistic character of nature

could be settled firmly if one used features of the experiment presented here to generate entanglement between more than two spatially separated particles^{23,24}. □

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1. Bennett, C. H. *et al.* Teleporting an unknown quantum state via dual classic and Einstein-Podolsky-Rosen channels. *Phys. Rev. Lett.* **70**, 1895–1899 (1993).
2. Schrödinger, E. Die gegenwärtige Situation in der Quantenmechanik. *Naturwissenschaften* **23**, 807–812; 823–828; 844–849 (1935).
3. Bennett, C. H. Quantum information and computation. *Phys. Today* **48**(10), 24–30, October (1995).
4. Bennett, C. H., Brassard, G. & Ekert, A. K. Quantum Cryptography. *Sci. Am.* **267**(4), 50–57, October (1992).
5. Mattle, K., Weinfurter, H., Kwiat, P. G. & Zeilinger, A. Dense coding in experimental quantum communication. *Phys. Rev. Lett.* **76**, 4656–4659 (1996).
6. Kwiat, P. G. *et al.* New high intensity source of polarization-entangled photon pairs. *Phys. Rev. Lett.* **75**, 4337–4341 (1995).
7. Hagley, E. *et al.* Generation of Einstein-Podolsky-Rosen pairs of atoms. *Phys. Rev. Lett.* **79**, 1–5 (1997).
8. Schumacher, B. Quantum coding. *Phys. Rev. A* **51**, 2738–2747 (1995).
9. Clauser, J. F. & Shimony, A. Bell's theorem: experimental tests and implications. *Rep. Prog. Phys.* **41**, 1881–1927 (1978).
10. Greenberger, D. M., Horne, M. A. & Zeilinger, A. Multiparticle interferometry and the superposition principle. *Phys. Today* August, 22–29 (1993).
11. Tittel, W. *et al.* Experimental demonstration of quantum-correlations over more than 10 kilometers. *Phys. Rev. Lett.* (submitted).
12. Zukowski, M., Zeilinger, A., Horne, M. A. & Ekert, A. "Event-ready-detectors" Bell experiment via entanglement swapping. *Phys. Rev. Lett.* **71**, 4287–4290 (1993).
13. Bose, S., Vedral, V. & Knight, P. L. A multiparticle generalization of entanglement swapping. preprint.
14. Wootters, W. K. & Zurek, W. H. A single quantum cannot be cloned. *Nature* **299**, 802–803 (1982).
15. Loudon, R. *Coherence and Quantum Optics VI* (eds Eberly, J. H. & Mandel, L.) 703–708 (Plenum, New York, 1990).
16. Zeilinger, A., Bernstein, H. J. & Horne, M. A. Information transfer with two-state two-particle quantum systems. *J. Mod. Optics* **41**, 2375–2384 (1994).
17. Weinfurter, H. Experimental Bell-state analysis. *Europhys. Lett.* **25**, 559–564 (1994).
18. Braunstein, S. L. & Mann, A. Measurement of the Bell operator and quantum teleportation. *Phys. Rev. A* **51**, R1727–R1730 (1995).
19. Michler, M., Mattle, K., Weinfurter, H. & Zeilinger, A. Interferometric Bell-state analysis. *Phys. Rev. A* **53**, R1209–R1212 (1996).
20. Zukowski, M., Zeilinger, A. & Weinfurter, H. Entangling photons radiated by independent pulsed sources. *Ann. NY Acad. Sci.* **755**, 91–102 (1995).
21. Fry, E. S., Walther, T. & Li, S. Proposal for a loophole-free test of the Bell inequalities. *Phys. Rev. A* **52**, 4381–4395 (1995).
22. Bennett, C. H. *et al.* Purification of noisy entanglement and faithful teleportation via noisy channels. *Phys. Rev. Lett.* **76**, 722–725 (1996).
23. Greenberger, D. M., Horne, M. A., Shimony, A. & Zeilinger, A. Bell's theorem without inequalities. *Am. J. Phys.* **58**, 1131–1143 (1990).
24. Zeilinger, A., Horne, M. A., Weinfurter, H. & Zukowski, M. Three particle entanglements from two entangled pairs. *Phys. Rev. Lett.* **78**, 3031–3034 (1997).

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Genomic sequence of a Lyme disease spirochaete, *Borrelia burgdorferi*

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The genome of the bacterium *Borrelia burgdorferi* B31, the aetiologic agent of Lyme disease, contains a linear chromosome of 910,725 base pairs and at least 17 linear and circular plasmids with a combined size of more than 533,000 base pairs. The chromosome contains 853 genes encoding a basic set of proteins for DNA replication, transcription, translation, solute transport and energy metabolism, but, like *Mycoplasma genitalium*, it contains no genes for cellular biosynthetic reactions. Because *B. burgdorferi* and *M. genitalium* are distantly related eubacteria, we suggest that their limited metabolic capacities reflect convergent evolution by gene loss from more metabolically competent progenitors. Of 430 genes on 11 plasmids, most have no known biological function; 39% of plasmid genes are paralogues that form 47 gene families. The biological significance of the multiple plasmid-encoded genes is not clear, although they may be involved in antigenic variation or immune evasion.

In the mid-1970s, a geographic clustering of an unusual rheumatoid arthritis-like condition was reported in Connecticut¹. That cluster of cases focused attention on the syndrome that is now called Lyme disease. It was subsequently realized that a similar disorder had been known in Europe since the beginning of this century. Lyme disease is characterized by some or all of the following manifestations: an initial erythematous annular rash, flu-like symptoms, neurological complications, and arthritis in about 50% of untreated patients². In the United States, the disease occurs primarily in northeastern and midwestern states, and in western parts of California and Oregon. These regions coincide with the ranges of various species of *Ixodes* ticks, the primary vector of Lyme disease. Lyme disease is now the most common tick-transmitted illness in the United States, and has been reported in many temperate parts of the Northern Hemisphere.

It was not until the early 1980s that a new spirochaete, *Borrelia burgdorferi*³, was isolated and cultured from the midgut of *Ixodes* ticks, and subsequently from patients with Lyme disease^{4,5}. Analysis of genetic diversity among individual *Borrelia* isolates has defined a closely related cluster containing at least 10 tick-borne species of Lyme disease agents, called '*B. burgdorferi* (*sensu lato*)'. *B. burgdorferi* resembles most other spirochaetes in that it is a highly specialized, motile, two-membrane, spiral-shaped bacterium that lives primarily as an extracellular pathogen. *Borrelia* is fastidious and difficult to culture *in vitro*, requiring a specially enriched media and low oxygen tension⁶.

One of the most striking features of *B. burgdorferi* is its unusual genome, which includes a linear chromosome approximately one megabase in size^{7–10} and numerous linear and circular plasmids^{11–13}, with some isolates containing up to 20 different plasmids. The plasmids have a copy number of approximately one per chromosome^{10,14}, and different plasmids often appear to share regions of homologous DNA^{13,15,16}. Long-term culture of *B. burgdorferi* results in the loss of some plasmids, changes in protein expression profiles,

and a loss in the ability of the organism to infect laboratory animals, suggesting that the plasmids encode important proteins involved in virulence^{17–19}.

Because of its importance as a pathogen of humans and animals, and the value of complete genome sequence information for understanding its life cycle and advancing drug and vaccine development, we sequenced the genome of *B. burgdorferi* type strain (B31), using the random sequencing method previously described^{20–24}. Here we summarize the results from sequencing, assembly and analysis of the linear chromosome and 11 plasmids.

Chromosome analysis

The linear chromosome of *B. burgdorferi* has 910,725 base pairs (bp) and an average G+C content of 28.6%. Base pair one represents the first double-stranded base pair that we observed at the left telomere. Previous genome characterizations agree with the nucleotide sequence of the large chromosome^{10,25–28}. The 853 predicted coding sequences (open reading frames; ORFs) have an average size of 992 bp, similar to that observed in other prokaryotic genomes, with 93% of the *B. burgdorferi* genome representing

Figure 1 Linear representations of the *B. burgdorferi* B31 chromosome and plasmids. The location of predicted coding regions colour-coded by biological role, RNA genes, and tRNAs is indicated. Arrows represent the direction of transcription for each predicted coding region. Numbers associated with tRNA symbols represent the number of tRNAs at a locus. Numbers associated with GES represent the number of membrane-spanning domains according to the Goldman, Engelman and Steitz scale as calculated by TopPred⁴⁹. Only proteins with five or more GES are indicated. Members of paralogous gene families are identified by family number. Transporter abbreviations: mal, maltose; P, gly and bet, proline, glycine, betaine; glyc, glycerol; aa, amino acid; E, glutamate; fru, fructose; glu, glucose; s/p, spermidine/putrescine; pan, pantothenate; Pi, phosphate; lac, lactate; rib, ribose; ?, unknown.

coding sequence. Biological roles were assigned to 59% of the 853 ORFs using the classification scheme adapted from Riley²⁹ (Fig. 1), 12% of ORFs matched hypothetical coding sequences of unknown function from other organisms, and 29% were new genes. The average relative molecular mass (M_r) of the chromosome-encoded proteins in *B. burgdorferi* is 37,529 ranging from 3,369 to 254,242, values similar to those observed in other bacteria including *Haemophilus influenzae*²⁰ and *Mycoplasma genitalium*²¹. The median isoelectric point (pI) for all predicted proteins is 9.7.

Analysis of codon usage in *B. burgdorferi* reveals that all 61 triplet codons are used. When both AU- and GC-containing codons specify a single amino acid, there is a marked bias (from 2-fold to more than 20-fold, depending on the amino acid) in the use of AU-rich codons. The most frequently used codons are AAA (Lys, 8.1%), AAU (Asn, 5.9%), AUU (Ile, 5.9%), UUU (Phe, 5.7%), GAA (Glu, 5.0%), GAU (Asp, 4.2%) and UUA (Leu, 4.2%). The most common amino acids are Ile (10.6%), Leu (10.3%), Lys (10.2%), Ser (7.8%) and Asn (7.2%). The high value for Lys is in agreement with the median calculated isoelectric point of 9.7.

Plasmid analysis

Analysis of the nucleotide sequence and Southern analyses on *B. burgdorferi* DNA indicate that, in addition to the large linear chromosome, isolate B31 contains linear plasmids of the following approximate sizes: 56 kilobase pairs (kbp) (lp56), 54 kbp (lp54), four plasmids of 28 kbp (lp28-1, lp28-2, lp28-3 and lp28-4), 38 kbp (lp38), 36 kbp (lp36), 25 kbp (lp25) and 17 kbp (lp17); and circular plasmids of the following sizes: 9 kbp (cp9), 26 kbp (cp26) and five or six homologous plasmids of 32 kbp (cp32). These include all of the plasmids previously identified in this strain, but comparisons with other B31 cultures suggest that this isolate may have lost one 21 kbp linear and one or two 32 kbp circular plasmids during growth in culture since its original isolation^{11–14,19,30}. The sequences of all plasmids were assembled as part of this project. However, the assembled sequences of the cp32 and related lp56 plasmids could not be determined with a high degree of confidence because of DNA sequence similarity among them ($\geq 99\%$ in several regions of

3,000–5,000 bp per plasmid)^{13,16} (Table 1). Improved assembly strategies are being tested to achieve closure on these plasmids (G. Sutton, unpublished). Plasmid lp17 is identical to that of lp16.9 from Barbour *et al.*¹⁵.

The 11 plasmids we have described contain a total of 430 putative ORFs with an average size of 507 bp; plasmid G+C content ranges from 23.1% to 32.3%. Only 71% of plasmid DNA represents predicted coding sequences, a value significantly lower than that on the chromosome. This indicates that average intergenic distances are greater in the plasmids than in the chromosome, and that many potential ORFs contain authentic frameshifts or stops (see E29, for example), suggesting that they are decaying genes not encoding functional proteins. Of the 430 plasmid ORFs, only 70 (16%) could be identified and these include membrane proteins such as OspA-D, decorin-binding proteins, the VlsE lipoprotein recombination cassette, and the purine ribonucleotide biosynthetic enzymes GuaA and GuaB. We found that 100 ORFs (23%) match other hypothetical proteins from plasmids in this and related strains of *B. burgdorferi*^{15,16,31}; 10 ORFs (2.3%) match hypothetical proteins from species other than *Borrelia*; and 250 ORFs (58%) have no database match.

We found that 47 paralogous gene families containing from 2 to 12 members account for 39% (169 ORFs) of the plasmid-encoded genes with no known biological role (Fig. 1). Parologue families 32 and 50, typified by previously identified *B. burgdorferi* plasmid genes cp32 orfC and cp8.3 orf2, respectively, have some similarities to proteins involved in replication, segregation and control of copy number in other bacterial systems^{16,31}. Previous studies have reported examples of plasmid gene duplication, but the extent of

Table 1 Genome features in *Borrelia burgdorferi*

Chromosome	910,725 bp (28.6% G+C)
Coding sequences (93%)	
RNAs (0.7%)	
Intergenic sequence (6.3%)	
853 coding sequences	
500 (59%) with identified database match	
104 (12%) match hypothetical proteins	
249 (29%) with no database match	
Plasmids	
cp9	9,386 bp (23.6% GC)
cp26	26,497 bp (26.3% GC)
lp17	16,828 bp (23.1% GC)
lp25	24,182 bp (23.3% GC)
lp28-1	26,926 bp (32.3% GC)
lp28-2	29,771 bp (31.5% GC)
lp28-3	28,605 bp (25.1% GC)
lp28-4	27,329 bp (24.4% GC)
lp36	36,834 bp (26.8% GC)
lp38	38,853 bp (26.1% GC)
lp54	53,590 bp (28.1% GC)
Coding sequences (71%)	
Intergenic sequence (29%)	
430 coding sequences	
70 (16%) with identified database match	
110 (26%) match hypothetical proteins	
250 (58%) with no database match	
Ribosomal RNA	Chromosome coordinates
16S	444581–446118
23S	438590–441508
5S	438446–438557
23S	435334–438267
5S	435201–435312
Stable RNA	
tmRNA	46973–47335
mpB	750816–751175
Transfer RNA	
34 species (8 clusters, 14 single genes)	

*The telomeric sequences of the nine linear plasmids assembled as part of this study were not determined; estimation of the number of missing terminal nucleotides by restriction analysis suggests that less than 1,200 bp is missing in all cases. Comparisons with previously determined sequences of lp 16.9 and one terminus of lp28-1 indicate that 25, 60 and 1,200 bp are missing, respectively.

Table 2 Gene identification numbers are listed with the prefix BB as in Fig. 2. Each gene identified is listed in its functional role category (adapted from Riley²⁹). The percentage of similarity and a two-letter abbreviation for genus and species for the best match are also shown. An expanded version of this table with additional information is available on the World-Wide Web at <http://www.tigr.org/tdb/mdb/bbdb/bbdb.htm>. Abbreviations of gene names are: Ac, acetyl; BP, binding protein; biosyn, biosynthesis; cello, cellobiose; CPDase, carboxypeptidase; Dcase, decarboxylase; DHase, dehydrogenase; flgr, flagellar/flagellum; fru, fructose; GBP, glycine, betaine, L-proline; glu, glucose; Kase, kinase; mal, maltose; MC-methyl-accepting chemotaxis; MTase, methyltransferase; NAG, N-acetylglucosamine; OH, hydroxy; OP, oligopeptide; P, phosphate; PPTase, phosphotransferase; PPase, phosphatase; prt, protein; put, putative; RDase, reductase; RG, ribose/galactose; SAM, S-adenosyl-methionine; Sase, synthetase/synthase; SP, spermidine/putrescine; ss, single-stranded; sub, subunit; Tase, transferase.

Abbreviation of genus and species are: Ah, *Aeromonas hydrophila*; Ar, *Agrobacterium radiobacter*; Al, *Alteromonas* sp.; Ab, *Anabaena* sp.; An, *Anacystis nidulans*; At, *Arabidopsis thaliana*; Av, *Azotobacter vinelandii*; Bf, *Bacillus firmus*; Bl, *Cacillus licheniformis*; Bm, *Bacillus megaterium*; Bs, *Bacillus stearothermophilus*; Bc, *Bacillus subtilis*; Bb, *Borrelia burgdorferi*; Bc, *Borrelia coriaceae*; Bh, *Borrelia hermsii*; Ba, *Buchnera aphidicola*; Ca, *Clostridium acetobutylicum*; Cl, *Clostridium longisporum*; Cp, *Clostridium perfringens*; Cg, *Corynebacterium glutamicum*; Cb, *Coxiella burnetii*; Cp, *Cyanophora paradoxa*; Dd, *Dictyostelium discoideum*; Ec, *Escherichia coli*; Eh, *Entamoeba histolytica*; Ee, *Enterobacter cloacae*; El, *Enterococcus faecalis*; Eh, *Enterococcus hirae*; Ha, *Haemophilus aegyptius*; Hi, *Haemophilus influenzae*; Hp, *Helicobacter pylori*; Hs, *Homo sapiens*; La, *Lactobacillus acidophilus*; Ll, *Lactococcus lactis*; Li, *Leptospira interrogans* serovar lai; Mj, *Methanococcus jannaschii*; Mb, *Methanosarcina barkeri*; Ml, *Mycobacterium leprae*; Mt, *Mycobacterium tuberculosis*; Mc, *Mycoplasma capricolum*; Mg, *Mycoplasma genitalium*; Mh, *Mycoplasma hominis*; Mh, *Mycoplasma hyorhinis*; Mm, *Mycoplasma mycoides*; Mp, *Mycoplasma pneumoniae*; Mx, *Mycoplasma xanthus*; Ng, *Neisseria gonorrhoeae*; Nm, *Neisseria meningitidis*; Os, *Odontella sinensis*; Pt, *Paramecium tetraurelia*; Pa, *Pediococcus acidilactici*; Pf, *Plasmodium falciparum*; Pg, *Porphyromonas gingivalis*; Pv, *Proteus vulgaris*; Pa, *Pseudomonas aeruginosa*; Pm, *Pseudomonas mevalonii*; Pp, *Pseudomonas putida*; Rm, *Rhizobium meliloti*; Rc, *Rhodobacter capsulatus*; Rs, *Rhodobacter sphaeroides*; Rp, *Rickettsia prowazekii*; Sc, *Saccharomyces cerevisiae*; Sc, *Salmonella choleraesuis*; St, *Salmonella typhimurium*; Sh, *Serpulina hyodysenteriae*; Sd, *Shigella dysenteriae*; So, *Spinacia oleracea*; Sc, *Staphylococcus carnosus*; Se, *Staphylococcus epidermidis*; Sp, *Streptococcus pyogenes*; Sc, *Streptomyces coelicolor*; Ss, *Sulfolobus solfataricus*; Syn, *Synechococcus* sp.; Sp, *Synechocystis* PCC6803; Tt, *Thermoplasma aerophilum*; Tm, *Thermoplasma acidophilum*; Tb, *Thermophilic bacterium* RT8.B4; Tv, *Thermoproteus tenax* virus; Tm, *Thermotoga maritima*; Tat, *Thermus aquaticus thermophilus*; Ta, *Thermus aquaticus*; Td, *Treponema denticola*; Tp, *Treponema pallidum*; Ta, *Triticum aestivum*; Tb, *Trypanosoma brucei* mitochondrion; Vc, *Vibrio cholerae*; Vp, *Vibrio parahaemolyticus*; Zm, *Zymomonas mobilis*.

this redundancy has become even more apparent with the complete sequence of these 11 plasmids from isolate B31. Moreover, a preliminary search of 221 putative ORFs from the cp32s and lp56 indicates that at least 50% display $\geq 70\%$ amino-acid similarity to ORFs from the other 11 plasmids presented here (data not shown). Although plasmid-encoded genes have been implicated in infectivity and virulence^{17–19}, the biological roles of most of these genes are not known. The significance of the large number of paralogous plasmid-encoded genes is not understood. These proteins may be expressed differentially in tick and mammalian hosts, or may undergo homologous recombination to generate antigenic variation in surface proteins. This hypothesis is supported by the identification of 63 plasmid-encoded putative membrane lipoproteins (Fig. 1).

Several copies of a putative recombinase/transposase similar to IS891-like transposases were identified in the *B. burgdorferi* plasmids. Linear plasmid 28-2 contains one full-length copy of this gene. Although no inverted repeats were found on either side of the transposase, there is a putative ribosome-binding site several nucleotides upstream of the apparent start codon, and a stem-loop structure ($-27 \text{ kcal mol}^{-1}$) 195 bp downstream of the stop codon in an area with no ORFs. This transposase might represent a functional gene important for the frequent DNA rearrangements that presumably occur in *Borrelia* plasmids. There are other partial or nearly complete copies of the transposase gene that contain frame-destroying mutations elsewhere in the genome: two copies on lp17, one on lp36, one on lp38, one on lp28-3, two on lp28-1, and one near the right end of the large linear chromosome.

Origin of replication

The replication mechanism for the linear chromosome and plasmids in *B. burgdorferi* is not yet known. Replication possibly begins at the termini, as has been proposed for the poxvirus hairpin telomeres³², or may begin from a single origin somewhere along the length of the linear replicon. Of the genes on the linear chromosome, 66% are transcribed away from the centre of the chromosome (Fig. 1), similar to the transcriptional bias observed for the genomes of *M. genitalium*²¹ and *M. pneumoniae*³³. It has been suggested that bacterial genes are optimally transcribed in the same direction as that in which replication forks pass over them, particularly for highly transcribed genes^{34,35}.

Given the transcriptional bias observed in *B. burgdorferi*, it seems likely that the origin of replication is near the centre of the chromosome. Because bacterial chromosomal replication origins are usually near *dnaA*³⁶, it is intriguing to note that this gene (BB437) lies almost exactly at the centre of the linear *B. burgdorferi* chromosome^{10,27}. A centrally initiated, bi-directional replication fork would be equidistant from the two chromosome ends, and replication would traverse the rRNA genes in the same direction as transcription.

An analysis of GC skew, $(G - C)/(G + C)$ calculated in 10-kilobase (kb) windows across the chromosome, shows a clear break at

the putative origin of replication. The GC-skew values are uniformly negative from 0 to 450 kb (minus strand), and uniformly positive (plus strand) from 450 kb to the end of the chromosome (Fig. 2). Additional evidence for the location of the origin of replication comes from our discovery of an octamer, TTGTTTTT, whose skewed distribution in the plus versus the minus strand of the chromosome matches the GC skew (Fig. 2). The biological significance of this octamer has not yet been determined, although it may be analogous to the Chi site in *Escherichia coli* that is implicated in *recBCD* mediated recombination. No GC skew was observed in any of the plasmids, although the heptamer ATTTT displays a skewed distribution in the plus versus the minus strand of lp28-4 that changes at the approximate midpoint of the plasmid (not shown).

Transcription and translation

Genes encoding the three subunits (α , β , β') of the core RNA polymerase were identified in *B. burgdorferi* along with σ^{70} and two alternative σ factors, σ^{54} and *rpoS*. The role and specificity of each of these σ factors in transcription regulation in *B. burgdorferi* are not known. The *nusA*, *nusB* and *rho* genes, which are involved in transcription elongation and termination, were also identified.

A region of the genome with a significantly higher G + C content (43%), located between nucleotides 434,000 and 447,000, contains the rRNA operon. As previously reported, the rRNA operon in *B. burgdorferi* contains a 16S rRNA–Ala-tRNA–Ile-tRNA–23S rRNA–5S rRNA–23S rRNA–5S rRNA^{37,38}. All of the genes are present in the same orientation, except for that encoding Ile tRNA. Four unrelated genes, encoding 3-methyladenine glycosylase, hydrolyase and two with no database match, are also present in the rRNA operon. Three of these genes are transcribed in the same direction as the rRNAs.

We identified in the chromosome 31 tRNAs with specificity for all 20 amino acids (Fig. 1). These are organized into 7 clusters plus 13 single genes. All tRNA synthetases are present except glutamyl tRNA-synthetase. A single glutamyl tRNA synthetase probably aminoacylates both tRNA^{Glu} and tRNA^{Gln} with glutamate followed by transamidation by Glu-tRNA amidotransferase, a heterotrimeric enzyme present in *B. burgdorferi* and several Gram-positive bacteria and archaea³⁰. The lysyl-tRNA synthetase (LysS) in *B. burgdorferi* is a class I type that has no resemblance to any known bacterial or eukaryotic LysS, but is most similar to LysS from the archaea⁴⁰.

Replication, repair and recombination

The complement of genes in *B. burgdorferi* involved in DNA replication is smaller than in *E. coli*, but similar to that in *M. genitalium*²¹. Three ORFs have been identified with high homology to four of the ten polypeptides in the *E. coli* DNA polymerase III: α , β and γ , and τ . In *E. coli*, the γ and τ proteins are produced by programmed ribosomal frameshifting. This observation suggests that DNA replication in *B. burgdorferi*, like that in *M. genitalium*, is accomplished with a restricted set of genes. *B. burgdorferi* has one

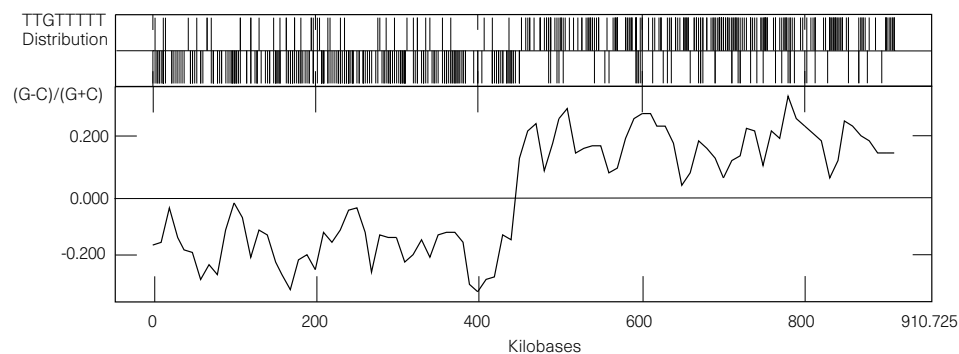


Figure 2 Distribution of TTGTTTTT and GC skew in the *B. burgdorferi* chromosome. Top, distribution of the octamer TTGTTTTT. The lines in the top panel represent the location of this octamer in the plus strand of the sequence, and those in the second panel represent the location of this oligomer in the minus strand of the sequence. Bottom, GC skew.

type I topoisomerase (*topA*) and two type II topoisomerases (gyrase and topoisomerase IV) for DNA topology management and chromosome segregation, despite its linear chromosomal structure. This suggests that topoisomerase IV may be required for more than the separation of circular DNAs during segregation.

The DNA repair mechanisms in *B. burgdorferi* are similar to those in *M. genitalium*. DNA excision repair can presumably occur by a pathway involving endonuclease III, PolI and DNA ligase. The genes for two of three DNA mismatch repair enzyme (*mutS*, *mutL*) are

present. The apparent absence of *mutH* is consistent with the lack of GATC (*dam*) methylation in strain B31 (S. Casjens, unpublished). Also present are genes for the repair of ultraviolet-induced DNA damage (*uvrA*, *uvrB*, *uvrC* and *uvrD*) (Table 2).

B. burgdorferi has a complete set of genes to perform homologous recombination, including *recA*, *recBCD*, *sbcC*, *sbcD*, *recG*, *ruvAB* and *recJ*. 3'-Exonuclease activity associated with *sbcB* in *E. coli* may be encoded by *exoA* (exodeoxynuclease III). Although *recA* is present, we found no evidence for *lexA*, which encodes the repressor that

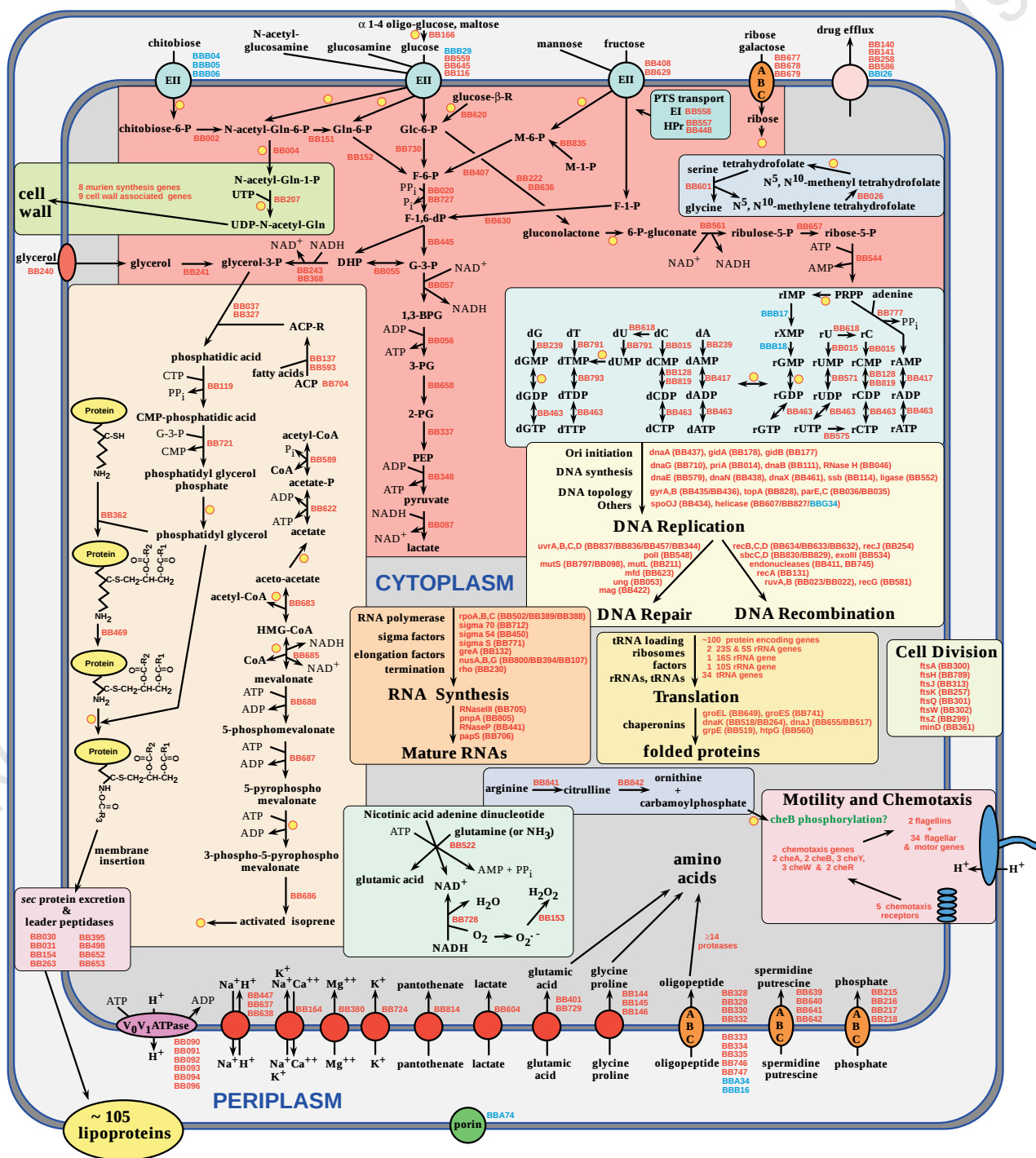


Figure 3 Solute transport and metabolic pathways in *B. burgdorferi*. A schematic diagram of a *B. burgdorferi* cell providing an integrated view of the transporters and the main components of the metabolism of this organism, as deduced from the genes identified in the genome. The ORF numbers correspond to those listed

in Table 2 (red indicates chromosomal and blue indicates plasmid ORFs). Presumed transporter specificity is indicated. Yellow circles indicate: places where particular uncertainties exist as to the substrate specificity, subcellular location or direction of catalysis: or expected activities that were not found.

regulates SOS genes in *E. coli*. No genes encoding DNA restriction or modification enzymes are present.

Biosynthetic pathways

The small genome size of *B. burgdorferi* is associated with an apparent absence of genes for the synthesis of amino acids, fatty acids, enzyme cofactors, and nucleotides, similar to that observed with *M. genitalium*²¹ (Fig. 3, Table 2). The lack of biosynthetic pathways explains why growth of *B. burgdorferi* *in vitro* requires serum-supplemented mammalian tissue-culture medium. This is also consistent with previous biochemical data indicating that *Borrelia* lack the ability to elongate long-chain fatty acids, such that the fatty-acid composition of *Borrelia* cells reflects that present in the growth medium⁶.

Transport

The linear chromosome of *B. burgdorferi* contains 46 ORFs and the plasmids contain 6 ORFs that encode transport and binding proteins (Fig. 3, Table 2). These gene products contribute to 16 distinct membrane transporters for amino acids, carbohydrates, anions and cations. The distribution of transporters between the four categories of functions in this section is similar to that observed in other heterotrophs (such as *Haemophilus influenzae*, *M. genitalium* and *H. pylori*), with most being dedicated to the import of organic compounds.

There are marked similarities between the transport capacity of *B.*

burgdorferi and *M. genitalium*. Both genomes have a limited number of recognizable transporters, so it is not clear how they can sustain diverse physiological reactions. Several of the identified transporters in both genomes exhibit broad substrate specificity, exemplified by the oligopeptide ABC transporter (*opp* operon) or the glycine, betaine, L-proline transport system (*proVWX*). Therefore, these organisms probably compensate for their restricted coding potential by producing proteins that can import a wide variety of solutes. This is important because *B. burgdorferi* is unable to synthesize any amino acids *de novo*. We were unable to identify any transport systems for nucleosides, nucleotides, NAD/NADH or fatty acids, although they are likely to be present.

Glucose, fructose, maltose and disaccharides seem to be acquired by the phosphoenolpyruvate:phosphotransferase system (PTS). The two nonspecific components, enzyme 1 (*ptsI*) and Hpr (*ptsH*), are associated in one operon with an apparently glucose-specific, phosphohistidine-sugar phosphotransferase enzyme IIA (*crr*). Separate from this operon are four permeases (enzyme IIBC), *fruA* in two copies (fructose), *ptsG* (glucose) and *malX* (glucose/maltose) (Fig. 3, Table 2). The fructose-specific enzyme IIA is induced in the ORF with IIBC (*fruA*), as has been observed in *M. genitalium*⁴¹. Ribose may be imported by an ATP-binding cassette transporter (*rbsAC*). The *rbsAC* genes are transcribed in an operon with a methyl-accepting chemotaxis protein that may respond to β -galactosides, suggesting that movement of the organisms towards sugars may be coupled to the transport process.

Energy metabolism

The limited metabolic capacity of *B. burgdorferi* is similar to that found in *M. genitalium* (Fig. 3, Table 2). Genes encoding all of the enzymes of the glycolytic pathway were identified. Analysis of the metabolic pathway suggests that *B. burgdorferi* uses glucose as a primary energy source, although other carbohydrates, including glycerol, glucosamine, fructose and maltose, may be used in glycolysis. Pyruvate produced by glycolysis is converted to lactate, consistent with the microaerophilic nature of *B. burgdorferi*. Generation of reducing power occurs through the oxidative branch of the pentose pathway. None of the genes encoding proteins of the tricarboxylic acid cycle or oxidative phosphorylation were identified. The similarity in metabolic strategies of two distantly related, obligate parasites, *M. genitalium* and *B. burgdorferi*, suggests convergent evolutionary gene loss from more metabolically competent, distant progenitors.

Addition of N-acetylglucosamine (NAG) to culture medium is required for growth of *B. burgdorferi*⁶. NAG is incorporated into the cell wall, and may also serve as an energy source. The cp26 plasmid encodes a PTS cellobiose transporter homologue that could have specificity for the structurally similar compound chitobiose (di-N-acetyl-D-glucosamine). A gene product on the chromosome with sequence similarity to chitobiose (BB2) may convert chitobiose to NAG. *B. burgdorferi* can metabolize NAG to fructose-6-phosphate, which then can enter the glycolytic cycle through the action of N-acetylglucosamine-6-phosphate deacetylase and glucosamine-6-phosphate isomerase. NAG is the primary constituent of chitin, which makes up the tick cuticle⁶, and may be a source of carbohydrate for *B. burgdorferi* when it is associated with its tick host.

The parallels between *B. burgdorferi* and *M. genitalium* appear to extend to other aspects of their metabolism. Both organisms lack a respiratory electron transport chain, so ATP production must be accomplished by substrate-level phosphorylation. Consequently, membrane potential is established by the reverse reaction of the V_1V_0 -type ATP synthase, here functioning as an ATPase to expel protons from the cytoplasm (Fig. 3, Table 2). The ATP synthase genes in *B. burgdorferi* appear to be transcribed as part of a seven-gene operon. They are not typical of those usually found in eubacteria, more closely resembling the eukaryotic vacuolar (V-type) and archaeal (A-type) H^+ -translocating ATPases⁴², both in size

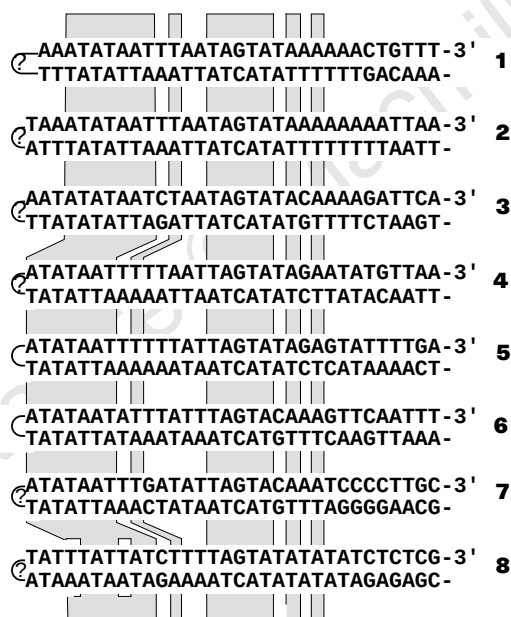


Figure 4 Telomere nucleotide sequences from *Borrelia* species. Nucleotide sequences are shown for known *Borrelia* telomeres as indicated: 1, *B. burgdorferi* Sh-2-82 chromosome left end; 2, *B. burgdorferi* B31 chromosome left end; 3, *B. afzelii* R-IP3 chromosome right end; 4, *B. burgdorferi* B31 chromosome right end; 5, *B. burgdorferi* B31 plasmid Ip17 left end; 6, *B. burgdorferi* B31 plasmid Ip17 right end; 7, *B. hermsii* plasmids bp7E and pb21E right ends; 8, *B. burgdorferi* B31 plasmid Ip28-1 right end. In each case the telomere is at the left. Question marks (?) indicate locations where S1 nuclease was used to open terminal hairpins during the sequence determinations. Stippled areas highlight regions that appear to have been most highly conserved among these telomeres; no strong sequence conservation has been found near the right of the terminal 26 bp among the different sequences listed, except between the chromosomal left ends from strains B31 and Sh-2-82 (see text). The telomeric sequences of the strain B31 chromosome were determined in this report; the others are from references 14, 28, 30, 45, 46.

and sequence similarity, than the bacterial F_1F_0 ATPases. Genome analysis of *Treponema pallidum*, the pathogenic spirochaete that causes syphilis, has also revealed the presence of a V_1V_0 -type ATP synthase (C. M. F. *et al.*, manuscript in preparation), suggesting that this may be a feature of spirochaetes.

Regulatory systems

Although the expression of *Borrelia* genes varies according to the current host species, temperature, host body location and other local factors, control of gene expression appears to differ from more well studied eubacteria. A typical set of homologues of heat-shock response genes is present (*groES*, *groEL*, *grpE*, *dnaJ*, *hslU*, *hslV*, *dnaK* and *htpG*), and *B. burgdorferi* is known to have such a response; however, it lacks the σ -32 that controls their transcription in *E. coli*. Only a few homologues to other eubacterial regulatory proteins are present, including only two response-regulator two-component systems.

Motility and chemotaxis

Like other spirochaetes, *B. burgdorferi* has periplasmic flagella that are inserted at each end of the cell and extend towards the middle of the cell body. The unique flagella allow the organism to move through viscous solutions, an ability that is presumed to be important in its migration to distant tissues following deposition in the skin layers⁴³. Proteins involved in motility and chemotaxis are encoded by 54 genes, more than 6% of the *B. burgdorferi* chromosome, most of which are arranged in eight operons containing between 2 and 25 genes.

B. burgdorferi contains several copies of the chemotaxis genes (*cheR*, *cheW*, *cheA*, *cheY* and *cheB*) downstream of the methyl-accepting chemotaxis proteins. Other eubacteria also have duplications of some *che* genes, but those genes in *B. burgdorferi* are the most redundant set yet found. *B. burgdorferi* lacks recognizable virulence factors; thus, its ability to migrate to distant sites in the tick and mammalian host is probably dependent on a robust chemotaxis response. Multiple chemotaxis genes may provide redundancy in this system in order to meet such challenges or, alternatively, these genes may be differentially expressed under varied physiological conditions. Another speculative possibility is that the flagellar motors at the two ends of the *B. burgdorferi* cell are different and require different *che* systems. In support of this idea is the observation that one of the motor switch genes, *fliG*, is also present in two copies.

Membrane protein analysis

Much of the previous work on *B. burgdorferi* has focused on outer-surface membrane genes because of their potential importance in bacterial detection and vaccination. Nearly all *Borrelia* membrane proteins have been found to be typical bacterial lipoproteins. A search of *B. burgdorferi* ORFs for a consensus lipobox in the first 30 amino acids identified 105 putative lipoproteins, representing more than 8% of coding sequences. This contrasts with a total of only 20 putative lipoproteins in the 1.67-million base pair *H. pylori* genome (1.3% of coding sequences)²³. The periplasmic binding proteins involved in transport of amino acids/peptides and phosphate in *B. burgdorferi* are candidate lipoproteins, suggesting that they may be anchored to the outer surface of the cytoplasmic membrane as in Gram-positive bacteria, rather than localized in the periplasmic space.

In better-characterized eubacteria, prolipoprotein diacylglycerol transferase (*lgt*), prolipoprotein signal peptidase (*lsp*), and apolipoprotein:phospholipid *N*-acyl transferase (*lnt*) are required for post-translational processing and addition of lipids to the amino-terminal cysteine. Genes for the first two of the enzymes (*lgt* and *lsp*) are present in the *B. burgdorferi* genome, but the gene for *lnt* was not identified, although biochemical evidence argues for all three activities in *B. burgdorferi*⁴⁴. The sequence similarity of an *lnt*

homologue in *B. burgdorferi* may be too low to be identified using our search methods, or its activity may be present in a new enzyme. In *E. coli* the Sec protein export system moves lipoproteins through the inner membrane, and *Borrelia* carries a complete set of these protein-secretion gene homologues (*secA/D/E/F/Y* and *tth*; only the non-essential *secB* is missing).

Analysis of telomeres

The two chromosomal telomeres of strain B31 have similar 26-bp inverted terminal sequences (Fig. 4). We found no other similarity between the two ends, and these 26-bp sequences are very similar to the previously characterized *Borrelia* telomeres. Terminal restriction fragments from both B31 chromosomal termini were shown to exhibit snapback kinetics (data not shown), strongly indicating that both terminate in covalently closed hairpins, like previously characterized *Borrelia* telomeres^{28,45,46}.

The left chromosomal telomere of strain B31 is identical to the previously characterized left telomere of strain Sh-2-82 (ref. 28), except for a 31 bp insertion in B31 26 bp from the end. The right-most 7,454 bp contains surprisingly few ORFs, given the ORF density elsewhere on the chromosome. The function of this region is unknown, but it contains several unusual features. The right terminal 900 bp contains considerable homology to the left ends of lp17 and lp28-3. The region between 3,600 bp and 8,000 bp from the right end also contains several areas with similarity to plasmid sequences, including a portion of the transposase-like gene approximately 4,500 bp from the right end. The spacing between the two conserved motifs (ATATAAT and TAGTATA) in the right 26-bp terminal repeat is the same as most previously known plasmid telomeres but different from the previously known chromosomal telomeres. These findings support the idea that the right end of the *Borrelia* chromosome has historically exchanged telomeres with the linear plasmids²⁸.

Conclusions

The *B. burgdorferi* genome sequence will provide a new starting point for the study of the pathogenesis, prevention and treatment of Lyme disease. With the exception of a small number of putative virulence genes (haemolysins and drug-efflux proteins), this organism contains few, if any, recognizable genes involved in virulence or host-parasite interactions, suggesting that *B. burgdorferi* differs from better-studied eubacteria in this regard. It will be interesting to determine the role of the multi-copy plasmid-encoded genes, as previous work has implicated plasmid genes in infectivity and virulence. The completion of the genome sequence from a second spirochaete, *Treponema pallidum* (C.M.F. *et al.*, manuscript in preparation) will allow for the identification of genes specific to each species and to this bacterial phylum, and will provide further insight into prokaryotic diversity. □

Methods

Cell lines. A portion of a low-passage subculture of the original Lyme-disease spirochaete tick isolate⁴ was obtained from A. Barbour. The type strain of *B. burgdorferi* (ATCC 35210)³, B31, was derived from this isolate by limiting dilution cloning⁵. Cells were grown in Barbour-Stoenner-Kelly medium II (BSKII)⁶, omitting the additions of antibiotics and gelatin, in tightly closed containers at 33–34 °C. Cells were subcultured three or fewer times *in vitro* between successive rounds of infection in C3H/HeJ mice to minimize loss of infectivity and plasmid content^{17,18}. After four successive transfers of infection in mice, a primary culture of B31, established from infected ear tissue, was expanded to 2.5 l by four successive subcultures. All available evidence indicates that the B31 line used for preparation of genomic DNA was probably clonal, as genetic heterogeneity was undetectable by several criteria including macrorestriction analysis (S. Casjens, unpublished data) and plasmid analysis of clonal derivatives of the B31 line¹³.

Sequencing. The *B. burgdorferi* genome was sequenced by a whole-genome random sequencing method previously applied to other microbial genomes^{20–24}.

An approximately 7.5-fold genome coverage was achieved by generating 19,078 sequences from a small insert plasmid library with an average edited length of 505 bases. The ends of 69 large insert lambda clones were sequenced to obtain a genome scaffold; 50% of the genome was covered by at least one lambda clone. Sequences were assembled using TIGR Assembler as described^{20–24}, resulting in a total of 524 assemblies containing at least two sequences, which were clustered into 85 groups based on linking information from forward and reverse sequence reads. All *Borrelia* sequences that had been mapped were searched against the assemblies in an attempt to delineate which were derived from the various elements of the *B. burgdorferi* genome. Some contigs were also located on the existing physical map by Southern analysis. Sequence and physical gaps for the chromosome were closed as described^{20–24}. At the completion of the project, less than 3% of the chromosome had single-fold coverage. The linear chromosome of *B. burgdorferi* has covalently closed hairpin structures at its termini that are similar to those reported for linear plasmids in this organism¹¹. The telomeric sequences (106 and 72 bp, respectively, from the left and right ends) were obtained after nicking the terminal loop with S1 nuclease and amplifying terminal sequences by ligation-mediated polymerase chain reaction (PCR) as described²⁸. The unknown terminal sequence was determined in both directions on four independent plasmid clones of the amplified DNA from each telomere. A minimum amount of S1 nuclease was used and, because of their sequence similarity to other *Borrelia* telomeres, it is likely that few, if any, nucleotides were lost from the B31 chromosomal telomeres in this process.

Identification of ORFs. Coding regions (ORFs) were identified using compositional analysis using an interpolated Markov model based on variable-length oligomers⁴⁷. ORFs of >600 bp were used to train the Markov model, as well as *B. burgdorferi* ORFs from GenBank. Once trained, the model was applied to the complete *B. burgdorferi* genome sequence and identified 953 candidate ORFs. ORFs that overlapped were visually inspected, and in some cases removed. Non-overlapping ORFs that were found between predicted coding regions and >30 amino acids in length were retained and included in the final annotation. All putative ORFs were searched against a non-redundant amino-acid database as described^{20–24}. ORFs were also analysed using 527 hidden Markov models constructed for several conserved protein families (PFAM v2.0) using HMMER⁴⁸. Families of paralogous genes were constructed by pairwise searches of proteins using FASTA. Matches that spanned at least 60% of the smaller of the protein pair were retained and visually inspected. A total of 94 paralogous gene families containing 293 genes were identified (Fig. 1).

Identification of membrane-spanning domains (MSDs). TopPred⁴⁹ was used to identify potential MSDs in proteins. A total of 526 proteins containing at least one putative MSD were identified, of which 183 were predicted to have more than one MSD. The presence of signal peptides and the probable position of a cleavage site in secreted proteins were detected using Signal-P as described²³; 189 proteins were predicted to have a signal peptide. Lipoproteins were identified by scanning for a lipobox in the first 30 amino acids of every protein. A consensus sequence relaxed from that used for *H. pylori*²³ was defined for the purpose of this search based on known or putative *B. burgdorferi* lipoprotein consensus sequences.

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1. Steere, A. C. et al. Lyme arthritis: an epidemic of oligoarticular arthritis in children and adults in three Connecticut communities. *Arthritis Rheum.* **20**, 7 (1977).
2. Steere, A. C. Lyme disease. *New Engl. J. Med.* **321**, 586–596 (1989).
3. Johnson, R. C., Schmidt, G. P., Hyde, F. W., Steigerwalt, A. G. & Brenner, D. J. *Borrelia burgdorferi* sp. nov.: etiologic agent of Lyme disease. *Int. J. Syst. Bacteriol.* **34**, 496–497 (1984).
4. Burgdorfer, W. et al. Lyme disease—a tick-borne spirochetosis? *Science* **216**, 1317–1319 (1982).
5. Barbour, A. G., Burgdorfer, W., Hayes, S. F., Peter, O. & Aeschlimann, A. Isolation of a cultivable spirochete from *Ixodes ricinus* ticks of Switzerland. *Curr. Microbiol.* **8**, 123–126 (1983).
6. Barbour, A. & Hayes, S. F. Biology of *Borrelia* species. *Microbiol. Rev.* **50**, 381–400 (1986).
7. Baril, C., Richaud, C., Baranton, G. & Saint Girons, I. Linear chromosome of *Borrelia burgdorferi*. *Res. Microbiol.* **140**, 507–516 (1989).
8. Ferdows, M. S. & Barbour, A. G. Megabase-sized linear DNA in the bacterium *Borrelia burgdorferi*, the Lyme disease agent. *Proc. Natl Acad. Sci. USA* **86**, 5969–5973 (1989).
9. Davidson, B., MacDougall, J. & Saint Girons, I. Physical map of the linear chromosome of the bacterium *Borrelia burgdorferi* 212, a causative agent of Lyme disease and localization of rRNA genes. *J. Bacteriol.* **174**, 3766–3774 (1992).
10. Casjens, S. & Huang, W. M. Linear chromosome physical and genetic map of *Borrelia burgdorferi*, the Lyme disease agent. *Mol. Microbiol.* **8**, 967–980 (1993).
11. Barbour, A. G. & Garon, C. F. Linear plasmids of the bacterium *Borrelia burgdorferi* have covalently closed ends. *Science* **237**, 409–411 (1987).
12. Barbour, A. G. Plasmid analysis of *Borrelia burgdorferi*, the Lyme disease agent. *J. Clin. Microbiol.* **26**, 475–478 (1988).

13. Casjens, S., van Vugt, R., Stevenson, B., Tilly, K. & Rosa, P. Homology throughout the multiple 32-kilobase circular plasmids present in Lyme disease spirochetes. *J. Bacteriol.* **171**, 217–227 (1997).
14. Hinnebusch, J. & Barbour, A. G. Linear- and circular-plasmid copy numbers in *Borrelia burgdorferi*. *J. Bacteriol.* **174**, 5251–5257 (1992).
15. Barbour, A. G., Carter, C. J., Bundoc, V. & Hinnebusch, J. The nucleotide sequence of a linear plasmid of *Borrelia burgdorferi* reveals similarities to those of circular plasmids of other prokaryotes. *J. Bacteriol.* **178**, 6625–6639 (1996).
16. Zuckert, W. R. & Meyer, J. Circular and linear plasmids of Lyme disease spirochetes share extensive homology: characterization of a repeated DNA element. *J. Bacteriol.* **178**, 2287–2298 (1996).
17. Schwan, T. G., Burgdorfer, W. & Garon, C. Changes in infectivity and plasmid profile of the Lyme disease spirochete, *Borrelia burgdorferi*, as a result of *in vitro* cultivation. *Infect. Immun.* **56**, 1831–1836 (1988).
18. Xu, Y., Kodner, C., Coleman, L. & Johnson, R. C. Correlation of plasmids with infectivity of *Borrelia burgdorferi sensu stricto* type strain B31. *Infect. Immun.* **64**, 3870–3876 (1996).
19. Norris, S. J., Howell, J. K., Garza, S. A., Ferdows, M. S. & Barbour, A. G. High- and low-infectivity phenotypes of clonal populations of *in vitro*-cultured *Borrelia burgdorferi*. *Infect. Immun.* **63**, 2206–2212 (1995).
20. Fleischmann, R. D. et al. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science* **269**, 496–512 (1995).
21. Fraser, C. M. et al. The minimal gene complement of *Mycoplasma genitalium*. *Science* **270**, 397–403 (1995).
22. Bult, C. J. et al. Complete genome sequence of the methanogenic archaeon, *Methanococcus jannaschii*. *Science* **273**, 1058–1072 (1996).
23. Tomb, J.-F. et al. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature* **388**, 539–547 (1997).
24. Klenk, H.-P. et al. The complete genome sequence of the hyperthermophilic, sulphate-reducing archaeon *Archaeoglobus fulgidus*. *Nature* **390**, 364–370 (1997).
25. Casjens, S., Ley, H., DeLange, M., Rosa, P. & Huang, W. Linear chromosomes of Lyme disease agent spirochetes: genetic diversity and conservation of gene order. *J. Bacteriol.* **177**, 2769–2780 (1995).
26. Casjens, S. & Huang, W. in *Bacterial Genomes: Physical Structure and Analysis* (eds de Bruijn, F., Lupski, J. & Weinstein, G. J.) (Chapman and Hall, New York, in the press).
27. Old, I., MacDougall, J., Saint Girons, I. & Davidson, B. Mapping of genes on the linear chromosome of the bacterium *Borrelia burgdorferi*: possible locations for its origin of replication. *FEMS Microbiol. Lett.* **99**, 245–250 (1992).
28. Casjens, S. et al. Telomeres of the linear chromosomes of Lyme disease spirochetes: nucleotide sequence and possible exchange with linear plasmid telomers. *Mol. Microbiol.* **26**, 581–596 (1997).
29. Riley, M. Functions of gene products of *Escherichia coli*. *Microbiol. Rev.* **57**, 862–952 (1993).
30. Zhang, J., Hardham, J., Barbour, A. & Norris, S. Antigenic variation in Lyme disease *Borreliae* by promiscuous recombination of VMP-like sequence cassettes. *Cell* **87**, 275–285 (1997).
31. Dunn, J. J. et al. Complete nucleotide sequence of a circular plasmid from the Lyme disease spirochete, *Borrelia burgdorferi*. *J. Bacteriol.* **176**, 2706–2717 (1994).
32. Traktman, P. in *DNA Replication in Eukaryotic Cells* (ed. DePamphilis, M.) 775–798 (Cold Spring Harbor Laboratory Press, NY, 1996).
33. Himmelreich, R. et al. Complete sequence analysis of the genome of the bacterium *Mycoplasma pneumoniae*. *Nucleic Acids Res.* **24**, 4420–4449 (1996).
34. Brewer, B. When polymerases collide: replication and the transcriptional organization of the *E. coli* chromosome. *Cell* **53**, 679–686 (1988).
35. French, S. Consequences of replication fork movement through transcription units *in vivo*. *Science* **258**, 1362–1365 (1992).
36. Ogasawara, N. & Yoshikawa, H. Genes and their organization in the replication origin region of the bacterial chromosome. *Mol. Microbiol.* **6**, 629–634 (1992).
37. Gazumyan, A., Schwartz, J. J., Liveris, D. & Schwartz, I. Sequence analysis of the ribosomal RNA operon of the Lyme disease spirochete, *Borrelia burgdorferi*. *Gene* **146**, 57–65 (1994).
38. Ojaimi, C., Davidson, B., Saint Girons, I. & Old, I. Conservation of gene arrangement and an unusual organization of rRNA genes in the linear chromosomes of Lyme disease spirochaetes *Borrelia burgdorferi*, *B. garinii* and *B. afzelii*. *Microbiology* **140**, 2931–2940 (1994).
39. Cumow, A. W. et al. Glu-tRNA^{Gln} amidotransferase: a novel heterotrimeric enzyme required for correct decoding of glutamine codons during translation. *Proc. Natl Acad. Sci. USA* **94**, 11819–11826 (1997).
40. Ibbat, M., Bobo, J. L., Rosa, P. A. & Soll, D. Archaeal-type lysyl-tRNA synthetase in the Lyme disease spirochete *Borrelia burgdorferi*. *Proc. Natl Acad. Sci. USA* (in the press).
41. Reizer, J., Paulsen, I. T., Reizer, A., Titzmeyer, F. & Saier, M. H. Jr Novel phosphotransferase system genes revealed by bacterial genome analysis: The complete complement of *pts* genes in *Mycoplasma genitalium*. *Microb. Comp. Genom.* **1**, 151–164 (1996).
42. Takase, K. et al. Sequencing and characterization of the *ntp* gene cluster for vacuolar-type Na⁺-translocating ATPase of *Enterococcus hirae*. *J. Biol. Chem.* **269**, 11037–11044 (1994).
43. Sadziane, A., Rosa, P. A., Thompson, P. A., Hogan, D. M. & Barbour, A. G. Antibody-resistant mutants of *Borrelia burgdorferi*: *in vitro* selection and characterization. *J. Exp. Med.* **176**, 799–809 (1992).
44. Brandt, M. E., Riley, B. S., Radolf, J. D. & Norgard, M. V. Immunogenic integral membrane proteins of *Borrelia burgdorferi* are lipoproteins. *Infect. Immun.* **58**, 983–991 (1990).
45. Hinnebusch, J., Bergstrom, S. & Barbour, A. Cloning and sequence analysis of linear plasmid telomeres of the bacterium *Borrelia burgdorferi*. *Mol. Microbiol.* **4**, 811–820 (1990).
46. Kitten, T. & Barbour, A. Juxtaposition of expressed variable antigen genes with a conserved telomere in the bacterium *Borrelia hermsii*. *Proc. Natl Acad. Sci. USA* **87**, 6077–6081 (1990).
47. Salzberg, S., Delcher, A., Kasif, S. & White, O. Microbial gene identification using interpolated Markov models. *Nucleic Acids Res.* (in the press).
48. Sonnhammer, E. L. L., Eddy, S. R. & Durbin, R. Pfam: a comprehensive database of protein families based on seed alignments. *Proteins* **28**, 405–420 (1997).
49. Claros, M. G. & von Heijne, G. TopPred II: an improved software for membrane proteins structure predictions. *Comput. Appl. Biosci.* **10**, 685–686 (1994).

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Correspondence and requests for materials should be sent to C.M.F. (e-mail: gbb@tigr.org). The annotated genome sequence and gene family alignments are available on the World-Wide Web at <http://www.tigr.org/tldb/mdb/bbdb/bbdb.html>. Sequences have been deposited with GenBank under the following accession numbers: AE00783 (chromosome); AE00784 (lp28-3); AE00785 (lp25); AE00786 (lp28-2); AE00787 (lp38); AE00788 (lp36); AE00789 (lp28-4); AE00790 (lp54); AE00791 (cp9); AE00792 (cp26); AE00793 (lp17); and AE00794 (lp28-1).

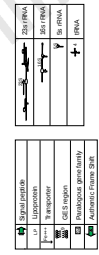
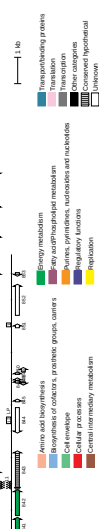


Table 2. Identification of *E. burgdorferi* genes

BB#	Identification (Species)	Genes	Accession	Function	BB#	Identification (Species)	Genes	Accession	Function
Animal and Microbial									
Swine family									
BB01	swine CHMTase (glyA) (Eo)	73	BBH20	outer membrane porin (omae%) prt (Eb)	74	BB291	tig basal-body rod prt (flF) (Eb)	100	
Biosynthesis of cofactors, prosthetic groups, and cofactors									
Folate									
BB02	methyltetrahydrofolate DHase (fdD) (Es)	61	BBH21	outer membrane porin (omae%) prt (Eb)	74	BB292	tig basal-body rod prt (flG) (Eb)	100	
Heme and porphyrin									
BB197	protoporphyrinogen oxidase prt (Es)	58	BBH22	outer membrane porin (omae%) prt (Eb)	74	BB293	tig basal-body rod prt (flH) (Eb)	100	
BB658	oxygen-independent coproporphyrinogen II oxidase prt (Es)	51	BBH23	outer membrane porin (omae%) prt (Eb)	74	BB294	tig basal-body rod prt (flI) (Eb)	100	
Menaquinone and ubiquinone									
BB314	coenzyme Q9 synthase (cpE) (Eo)	57	BBH24	outer membrane porin (omae%) prt (Eb)	74	BB295	tig basal-body rod prt (flJ) (Eb)	100	
Pantoic acid									
BB112	pantoic acid methyltransferase prt (Eb)	53	BBH25	outer membrane porin (omae%) prt (Eb)	74	BB296	tig basal-body rod prt (flK) (Eb)	100	
Pyridoxine									
BB768	pyridoxal kinase (pdxK) (Sc)	47	BBH26	outer membrane porin (omae%) prt (Eb)	74	BB297	tig basal-body rod prt (flL) (Eb)	100	
Thiamine									
BB21	4-methyl-5-(b-hydroxyethyl)-thiazole monophosphate prt (Hs)	53	BBH27	outer membrane porin (omae%) prt (Eb)	74	BB298	tig basal-body rod prt (flM) (Eb)	100	
Pyridine nucleotides									
BB522	NH ₄ ⁺ -dependent NAD ⁺ synthase (Fcd)	65	BBH28	outer membrane porin (omae%) prt (Eb)	74	BB299	tig basal-body rod prt (flN) (Eb)	100	
Cell envelope									
Membranes, lipoproteins, and porins									
BB332	basal membrane prt B (bnpB) (Eb)	100	BBH29	outer membrane porin (omae%) prt (Eb)	74	BB300	tig basal-body rod prt (flO) (Eb)	100	
BB333	basal membrane prt A (bnpA) (Eb)	100	BBH30	outer membrane porin (omae%) prt (Eb)	74	BB301	tig basal-body rod prt (flP) (Eb)	100	
BB334	basal membrane prt C (bnpC) (Eb)	100	BBH31	outer membrane porin (omae%) prt (Eb)	74	BB302	tig basal-body rod prt (flQ) (Eb)	100	
BB335	basal membrane prt D (bnpD) (Eb)	100	BBH32	outer membrane porin (omae%) prt (Eb)	74	BB303	tig basal-body rod prt (flR) (Eb)	100	
BB108	basal membrane prt (Tp)	50	BBH33	outer membrane porin (omae%) prt (Eb)	74	BB304	tig basal-body rod prt (flS) (Eb)	100	
BB319	exported prt (tpnB) (Tp)	50	BBH34	outer membrane porin (omae%) prt (Eb)	74	BB305	tig basal-body rod prt (flT) (Eb)	100	
BB347	transmembrane prt (tpnA) (Tp)	53	BBH35	outer membrane porin (omae%) prt (Eb)	74	BB306	tig basal-body rod prt (flU) (Eb)	100	
BB442	inner membrane prt (Hi)	63	BBH36	outer membrane porin (omae%) prt (Eb)	74	BB307	tig basal-body rod prt (flV) (Eb)	100	
BB345	lipoprotein Lx7 (Eb)	100	BBH37	outer membrane porin (omae%) prt (Eb)	74	BB308	tig basal-body rod prt (flW) (Eb)	100	
BB603	membrane-associated prt prt (Eb)	100	BBH38	outer membrane porin (omae%) prt (Eb)	74	BB309	tig basal-body rod prt (flX) (Eb)	100	
BB753	membrane opening prt prt (Sc)	40	BBH39	outer membrane porin (omae%) prt (Eb)	74	BB310	tig basal-body rod prt (flY) (Eb)	100	
BB755	outer membrane prt (Ng)	48	BBH40	outer membrane porin (omae%) prt (Eb)	74	BB311	tig basal-body rod prt (flZ) (Eb)	100	
BB167	outer membrane prt (tpnB) (Tp)	48	BBH41	outer membrane porin (omae%) prt (Eb)	74	BB312	tig basal-body rod prt (flA) (Eb)	100	
BB735	rare lipoprotein A (flA) (Hi)	53	BBH42	outer membrane porin (omae%) prt (Eb)	74	BB313	tig basal-body rod prt (flB) (Eb)	100	
BB10	surface-associated membrane prt 1 (bnp1) (Hi)	45	BBH43	outer membrane porin (omae%) prt (Eb)	74	BB314	tig basal-body rod prt (flC) (Eb)	100	
BB153	S2 prt (Eb)	60	BBH44	outer membrane porin (omae%) prt (Eb)	74	BB315	tig basal-body rod prt (flD) (Eb)	100	
Cell processes									
General									
BB54	chorion BP A (cbpA) (Eb)	94	BBH45	outer membrane porin (omae%) prt (Eb)	74	BB316	tig basal-body rod prt (flE) (Eb)	100	
BB424	chorion BP B (cbpB) (Eb)	100	BBH46	outer membrane porin (omae%) prt (Eb)	74	BB317	tig basal-body rod prt (flF) (Eb)	100	
BB425	chorion BP C (cbpC) (Eb)	54	BBH47	outer membrane porin (omae%) prt (Eb)	74	BB318	tig basal-body rod prt (flG) (Eb)	100	
BB426	lipoprotein (Eb)	100	BBH48	outer membrane porin (omae%) prt (Eb)	74	BB319	tig basal-body rod prt (flH) (Eb)	100	
BB427	lipoprotein (Eb)	100	BBH49	outer membrane porin (omae%) prt (Eb)	74	BB320	tig basal-body rod prt (flI) (Eb)	100	
BB428	lipoprotein (Eb)	100	BBH50	outer membrane porin (omae%) prt (Eb)	74	BB321	tig basal-body rod prt (flJ) (Eb)	100	
BB429	outer membrane porin (omae%) (Eb)	100	BBH51	outer membrane porin (omae%) prt (Eb)	74	BB322	tig basal-body rod prt (flK) (Eb)	100	
BB430	outer surface prt A (cpA) (Eb)	99	BBH52	outer membrane porin (omae%) prt (Eb)	74	BB323	tig basal-body rod prt (flL) (Eb)	100	
BB431	outer surface prt B (cpB) (Eb)	99	BBH53	outer membrane porin (omae%) prt (Eb)	74	BB324	tig basal-body rod prt (flM) (Eb)	100	
BB432	outer membrane prt (Eb)	100	BBH54	outer membrane porin (omae%) prt (Eb)	74	BB325	tig basal-body rod prt (flN) (Eb)	100	
BB433	outer surface prt C (cpC) (Eb)	99	BBH55	outer membrane porin (omae%) prt (Eb)	74	BB326	tig basal-body rod prt (flO) (Eb)	100	
BB434	outer surface prt D (cpD) (Eb)	99	BBH56	outer membrane porin (omae%) prt (Eb)	74	BB327	tig basal-body rod prt (flP) (Eb)	100	
BB435	outer surface prt E (cpE) (Eb)	99	BBH57	outer membrane porin (omae%) prt (Eb)	74	BB328	tig basal-body rod prt (flQ) (Eb)	100	
BB436	outer surface prt F (cpF) (Eb)	99	BBH58	outer membrane porin (omae%) prt (Eb)	74	BB329	tig basal-body rod prt (flR) (Eb)	100	
BB437	outer surface prt G (cpG) (Eb)	99	BBH59	outer membrane porin (omae%) prt (Eb)	74	BB330	tig basal-body rod prt (flS) (Eb)	100	
BB438	outer surface prt H (cpH) (Eb)	99	BBH60	outer membrane porin (omae%) prt (Eb)	74	BB331	tig basal-body rod prt (flT) (Eb)	100	
BB439	outer surface prt I (cpI) (Eb)	99	BBH61	outer membrane porin (omae%) prt (Eb)	74	BB332	tig basal-body rod prt (flU) (Eb)	100	
BB440	outer surface prt J (cpJ) (Eb)	99	BBH62	outer membrane porin (omae%) prt (Eb)	74	BB333	tig basal-body rod prt (flV) (Eb)	100	
BB441	outer surface prt K (cpK) (Eb)	99	BBH63	outer membrane porin (omae%) prt (Eb)	74	BB334	tig basal-body rod prt (flW) (Eb)	100	
BB442	outer surface prt L (cpL) (Eb)	99	BBH64	outer membrane porin (omae%) prt (Eb)	74	BB335	tig basal-body rod prt (flX) (Eb)	100	
BB443	outer surface prt M (cpM) (Eb)	99	BBH65	outer membrane porin (omae%) prt (Eb)	74	BB336	tig basal-body rod prt (flY) (Eb)	100	
BB444	outer surface prt N (cpN) (Eb)	99	BBH66	outer membrane porin (omae%) prt (Eb)	74	BB337	tig basal-body rod prt (flZ) (Eb)	100	
BB445	outer surface prt O (cpO) (Eb)	99	BBH67	outer membrane porin (omae%) prt (Eb)	74	BB338	tig basal-body rod prt (flA) (Eb)	100	
BB446	outer surface prt P (cpP) (Eb)	99	BBH68	outer membrane porin (omae%) prt (Eb)	74	BB339	tig basal-body rod prt (flB) (Eb)	100	
BB447	outer surface prt Q (cpQ) (Eb)	99	BBH69	outer membrane porin (omae%) prt (Eb)	74	BB340	tig basal-body rod prt (flC) (Eb)	100	
BB448	outer surface prt R (cpR) (Eb)	99	BBH70	outer membrane porin (omae%) prt (Eb)	74	BB341	tig basal-body rod prt (flD) (Eb)	100	
BB449	outer surface prt S (cpS) (Eb)	99	BBH71	outer membrane porin (omae%) prt (Eb)	74	BB342	tig basal-body rod prt (flE) (Eb)	100	
BB450	outer surface prt T (cpT) (Eb)	99	BBH72	outer membrane porin (omae%) prt (Eb)	74	BB343	tig basal-body rod prt (flF) (Eb)	100	
BB451	outer surface prt U (cpU) (Eb)	99	BBH73	outer membrane porin (omae%) prt (Eb)	74	BB344	tig basal-body rod prt (flG) (Eb)	100	
BB452	outer surface prt V (cpV) (Eb)	99	BBH74	outer membrane porin (omae%) prt (Eb)	74	BB345	tig basal-body rod prt (flH) (Eb)	100	
BB453	outer surface prt W (cpW) (Eb)	99	BBH75	outer membrane porin (omae%) prt (Eb)	74	BB346	tig basal-body rod prt (flI) (Eb)	100	
BB454	outer surface prt X (cpX) (Eb)	99	BBH76	outer membrane porin (omae%) prt (Eb)	74	BB347	tig basal-body rod prt (flJ) (Eb)	100	
BB455	outer surface prt Y (cpY) (Eb)	99	BBH77	outer membrane porin (omae%) prt (Eb)	74	BB348	tig basal-body rod prt (flK) (Eb)	100	
BB456	outer surface prt Z (cpZ) (Eb)	99	BBH78	outer membrane porin (omae%) prt (Eb)	74	BB349	tig basal-body rod prt (flL) (Eb)	100	
BB457	outer surface prt AA (cpAA) (Eb)	99	BBH79	outer membrane porin (omae%) prt (Eb)	74	BB350	tig basal-body rod prt (flM) (Eb)	100	
BB458	outer surface prt AB (cpAB) (Eb)	99	BBH80	outer membrane porin (omae%) prt (Eb)	74	BB351	tig basal-body rod prt (flN) (Eb)	100	
BB459	outer surface prt AC (cpAC) (Eb)	99	BBH81	outer membrane porin (omae%) prt (Eb)	74	BB352	tig basal-body rod prt (flO) (Eb)	100	
BB460	outer surface prt AD (cpAD) (Eb)	99	BBH82	outer membrane porin (omae%) prt (Eb)	74	BB353	tig basal-body rod prt (flP) (Eb)	100	
BB461	outer surface prt AE (cpAE) (Eb)	99	BBH83	outer membrane porin (omae%) prt (Eb)	74	BB354	tig basal-body rod prt (flQ) (Eb)	100	
BB462	outer surface prt AF (cpAF) (Eb)	99	BBH84	outer membrane porin (omae%) prt (Eb)	74	BB355	tig basal-body rod prt (flR) (Eb)	100	
BB463	outer surface prt AG (cpAG) (Eb)	99	BBH85	outer membrane porin (omae%) prt (Eb)	74	BB356	tig basal-body rod prt (flS) (Eb)	100	
BB464	outer surface prt AH (cpAH) (Eb)	99	BBH86	outer membrane porin (omae%) prt (Eb)	74	BB357	tig basal-body rod prt (flT) (Eb)	100	
BB465	outer surface prt AI (cpAI) (Eb)	99	BBH87	outer membrane porin (omae%) prt (Eb)	74	BB358	tig basal-body rod prt (flU) (Eb)	100	
BB466	outer surface prt AJ (cpAJ) (Eb)	99	BBH88	outer membrane porin (omae%) prt (Eb)	74	BB359	tig basal-body rod prt (flV) (Eb)	100	
BB467	outer surface prt AK (cpAK) (Eb)	99	BBH89	outer membrane porin (omae%) prt (Eb)	74	BB360	tig basal-body rod prt (flW) (Eb)	100	
BB468	outer surface prt AL (cpAL) (Eb)	99	BBH90	outer membrane porin (omae%) prt (Eb)	74	BB361	tig basal-body rod prt (flX) (Eb)	100	
BB469	outer surface prt AM (cpAM) (Eb)	99	BBH91	outer membrane porin (omae%) prt (Eb)	74	BB362	tig basal-body rod prt (flY) (Eb)	100	
BB470	outer surface prt AN (cpAN) (Eb)	99	BBH92	outer membrane porin (omae%) prt (Eb)	74	BB363	tig basal-body rod prt (flZ) (Eb)	100	
BB471	outer surface prt AO (cpAO) (Eb)	99	BBH93	outer membrane porin (omae%) prt (Eb)	74	BB364	tig basal-body rod prt (flA) (Eb)	100	
BB472	outer surface prt AP (cpAP) (Eb)	99	BBH94	outer membrane porin (omae%) prt (Eb)	74	BB365	tig basal-body rod prt (flB) (Eb)	100	
BB473	outer surface prt AQ (cpAQ) (Eb)	99	BBH95	outer membrane porin (omae%) prt (Eb)	74	BB366	tig basal-body rod prt (flC) (Eb)	100	
BB474	outer surface prt AR (cpAR) (Eb)	99	BBH96	outer membrane porin (omae%) prt (Eb)	74	BB367	tig basal-body rod prt (flD) (Eb)	100	
BB475	outer surface prt AS (cpAS) (Eb)	99	BBH97	outer membrane porin (omae%) prt (Eb)	74	BB368	tig basal-body rod prt (flE) (Eb)	100	
BB476	outer surface prt AT (cpAT) (Eb)	99	BBH98	outer membrane porin (omae%) prt (Eb)	74	BB369	tig basal-body rod prt (flF) (Eb)	100	
BB477	outer surface prt AU (cpAU) (Eb)	99	BBH99	outer membrane porin (omae%) prt (Eb)	74	BB370	tig basal-body rod prt (flG) (Eb)	100	
BB478	outer surface prt AV (cpAV) (Eb)	99	BBH100	outer membrane porin (omae%) prt (Eb)	74	BB371	tig basal-body rod prt (flH) (Eb)	100	
BB479	outer surface prt AW (cpAW) (Eb)	99	BBH101	outer membrane porin (omae%) prt (Eb)	74	BB372	tig basal-body rod prt (flI) (Eb)	100	
BB480	outer surface prt AX (cpAX) (Eb)	99	BBH102	outer membrane porin (omae%) prt (Eb)	74	BB373	tig basal-body rod prt (flJ) (Eb)	100	
BB481	outer surface prt AY (cpAY) (Eb)	99	BBH103	outer membrane porin (omae%) prt (Eb)	74	BB374	tig basal-body rod prt (flK) (Eb)	100	
BB482	outer surface prt AZ (cpAZ) (Eb)	99	BBH104	outer membrane porin (omae%) prt (Eb)	74	BB375	tig basal-body rod prt (flL) (Eb)	100	
BB483	outer surface prt BA (cpBA) (Eb)	99	BBH105	outer membrane porin (omae%) prt (Eb)	74	BB376	tig basal-body rod prt (flM) (Eb)	100	
BB484	outer surface prt BB (cpBB) (Eb)	99	BBH106	outer membrane porin (omae%) prt (Eb)	74	BB377	tig basal-body rod prt (flN) (Eb)	100	
BB485	outer surface prt BC (cpBC) (Eb)	99	BBH107	outer membrane porin (omae%) prt (Eb)	74	BB378	tig basal-body rod prt (flO) (Eb)	100	
BB486	outer surface prt BD (cpBD) (Eb)	99	BBH108	outer membrane porin (omae%) prt (Eb)	74	BB379	tig basal-body rod prt (flP) (Eb)	100	
BB487	outer surface prt BE (cpBE) (Eb)	99	BBH109	outer membrane porin (omae%) prt (Eb)	74	BB380	tig basal-body rod prt (flQ) (Eb)	100	
BB488	outer surface prt BF (cpBF) (Eb)	99	BBH110	outer membrane porin (omae%) prt (Eb)	74	BB381	tig basal-body rod prt (flR) (Eb)	100	
BB489	outer surface prt BG (cpBG) (Eb)	99	BBH111	outer membrane porin (omae%) prt (Eb)	74	BB382	tig basal-body rod prt (flS) (Eb)	100	
BB490	outer surface prt BH (cpBH) (Eb)	99	BBH112	outer membrane porin (omae%) prt (Eb)	74	BB383	tig basal-body rod prt (flT) (Eb)	100	
BB491	outer surface prt BI (cpBI) (Eb)	99	BBH113	outer membrane porin (omae%) prt (Eb)	74	BB384	tig basal-body rod prt (flU) (Eb)	100	
BB492	outer surface prt BJ (cpBJ) (Eb)	99	BBH114	outer membrane porin (omae%) prt (Eb)	74	BB385	tig basal-body rod prt (flV) (Eb)	100	
BB493	outer surface prt BK (cpBK) (Eb)	99	BBH115	outer membrane porin (omae%) prt (Eb)	74	BB386	tig basal-body rod prt (flW) (Eb)	100	
BB494	outer surface prt BL (cpBL) (Eb)	99	BBH116	outer membrane porin (omae%) prt (Eb)	74	BB387	tig basal-body rod prt (flX) (Eb)	100	
BB495	outer surface prt BM (cpBM) (Eb)	99	BBH117	outer membrane porin (omae%) prt (Eb)	74	BB388	tig basal-body rod prt (flY) (Eb)	100	
BB496	outer surface prt BN (cpBN) (Eb)	99	BBH118	outer membrane porin (omae%) prt (Eb)	74	BB389	tig basal-body rod prt (flZ) (Eb)	100	
BB497	outer surface prt BO (cpBO) (Eb)	99	BBH119	outer membrane porin (omae%) prt (Eb)	74	BB390	tig basal-body rod prt (flA) (Eb)	100	
BB498	outer surface prt BP (cpBP) (Eb)	99	BBH120	outer membrane porin (omae%) prt (Eb)	74	BB391	tig basal-body rod prt (flB) (Eb)	100	
BB499	outer surface prt BQ (cpBQ) (Eb)	99	BBH121	outer membrane porin (omae%) prt (Eb)	74	BB392	tig basal-body rod prt (flC) (Eb)	100	
BB500	outer surface prt BR (cpBR) (Eb)	99	BBH122	outer membrane porin (omae%) prt (Eb)	74	BB393	tig basal-body rod prt (flD) (Eb)	100	
BB501	outer surface prt BS (cpBS) (Eb)	99	BBH123	outer membrane porin (omae%) prt (Eb)	74	BB394	tig basal-body rod prt (flE) (Eb)	100	
BB502	outer surface prt BT (cpBT) (Eb)	99	BBH124	outer membrane porin (omae%) prt (Eb)	74	BB395	tig basal-body rod prt (flF) (Eb)	100	
BB503	outer surface prt BU (cpBU) (Eb)	99	BBH125	outer membrane porin (omae%) prt (Eb)	74	BB396	tig basal-body rod prt (flG) (Eb)	100	
BB504	outer surface prt BV (cpBV) (Eb)	99	BBH126	outer membrane porin (omae%) prt (Eb)	74	BB397	tig basal-body rod prt (flH) (Eb)	100	
BB505	outer surface prt BW (cpBW) (Eb)	99	BBH127	outer membrane porin (omae%) prt (Eb)	74	BB398	tig basal-body rod prt (flI) (Eb)	100	
BB506	outer surface prt BX (cpBX) (Eb)	99	BBH128	outer membrane porin (omae%) prt (Eb)	74	BB399	tig basal-body rod prt (flJ) (Eb)	100	
BB507	outer surface prt BY (cpBY) (Eb)	99	BBH129	outer membrane porin (omae%) prt (Eb)	74	BB400	tig basal-body rod prt (flK) (Eb)	100	
BB508	outer surface prt BZ (cpBZ) (Eb)	99	BBH130	outer membrane porin (omae%) prt (Eb)	74	BB401	tig basal-body rod prt (flL) (Eb)	100	
BB509	outer surface prt CA (cpCA) (Eb)	99	BBH131	outer membrane porin (omae%) prt (Eb)	74	BB402	tig basal-body rod prt (flM) (Eb)	100	
BB510	outer surface prt CB (cpCB) (Eb)	99	BBH132	outer membrane porin (omae%) prt (Eb)	74	BB403	tig basal-body rod prt (flN) (Eb)	100	
BB511	outer surface prt CC (cpCC) (Eb)	99	BBH133	outer membrane porin (omae%) prt (Eb)	74	BB404	tig basal-body rod prt (flO) (Eb)	100	
BB512	outer surface prt CD (cpCD) (Eb)	99	BBH134	outer membrane porin (omae%) prt (Eb)	74	BB405	tig basal-body rod prt (flP) (Eb)	100	
BB513	outer surface prt CE (cpCE) (Eb)	99	BBH135	outer membrane porin (omae%) prt (Eb)	74	BB406			

lp28-2			ATP-proton motive force interconversion	BB575	CTP Sase (pyrG) [Mj]	71
BBG08	stage 0 sporulation prt J (spoOJ) [Bb]	66	BB094 V-type ATPase, sub A (atpA) [Mb]	64	<i>Salvage of nucleosides and nucleotides</i>	
			BB093 V-type ATPase, sub B (atpB) [Mb]	62	adenine phosphoribosylTase (apt) [Ta]	63
<i>Cell killing</i>			BB092 V-type ATPase, sub D (atpD) [Mj]	51	BB618 cytidine deaminase (cdd) [Mp]	61
BB143	-hemolysin (hlyA) [Ah]	62	BB096 V-type ATPase, sub E (atpE) [Mj]	54	BB239 deoxyguanosine/deoxyadenosine kinase(I) sub 2 (dck) [La]	59
BB117	hemolysin III (yplQ) [Bs]	61	BB091 V-type ATPase, sub I (atpI) [Eh]	53	BB375 pfs prt (pfs-1) [Ec]	64
BB506	hemolysin (tlyA) [Sh]	59	BB090 V-type ATPase, sub K (atpK) [Mj]	54	BB588 pfs prt (pfs-2) [Hi]	59
BB059	hemolysin (tlyC) [Sh]	65			BB791 thymidine kinase (tdk) [Bs]	47
BB202	hemolysin, put [Syn]	54	<i>Electron transport</i>		BB015 uridine kinase (udk) [Bb]	100
			BB061 thioredoxin (trxA) [Ec]	59		
<i>Chaperones</i>			BB515 thioredoxin RDase (trxB) [Bb]	99		
BB741	chaperonin (groES) [Pg]	77	<i>Fermentation</i>			
BB602	chaperonin, put [Cb]	72	BB622 acetate kinase (ackA) [Ec]	63	lp36	
BB519	grpE prt (grpE) [Bb]	100	BB589 P AcTase (pta) [Tt]	65	BBK17	adenine deaminase (adeC) [Bs]
BB295	heat shock prt (hslU) [Bb]	100				
BB296	heat shock prt (hslV) [Bb]	100	<i>Glycolysis</i>		Regulatory functions	
BB649	heat shock prt (groEL) [Bb]	100	BB337 enolase (eno) [Bs]	79	<i>General</i>	
BB517	heat shock prt (dnaI-1) [Bb]	100	BB445 fructose-bisP aldolase (fba) [Ec]	80	BB184	carbon storage regulator (csrA) [Hi]
BB655	heat shock prt (dnaI-2) [Ca]	59	BB730 glucose-6-P isomerase (pgi) [Pf]	62		63
BB264	heat shock prt 70 (dnaK-1) [Bb]	61	BB057 glyceraldehyde 3-P DHase (gap) [Bb]	99	BB647	ferric uptake regulation prt (fur) [Sp]
BB518	heat shock prt 70 (dnaK-2) [Bb]	100	BB630 1-phosphofructoKase (fruK) [Hi]	52		48
BB560	heat shock prt 90 (htpG) [Bb]	100	BB056 phosphoglycerate Kase (pgk) [Bb]	99	BB198	guanosine-3',5'-bis(diP) 3'-pyrophosphohydrolase (spoT) [Ec]
			BB658 phosphoglycerate mutase (gpmA) [Ec]	79	BB737	histidine phosphoKase/PPase, put [Ml]
<i>Detoxification</i>			BB348 pyruvate Kase (pyk) [Bs]	62		49
BB153	superoxide dismutase (sodA) [Hi]	68	BB727 pyroP-fructose 6-P 1-PPTase (pfk) [Eh]	65	BB176	melanin DHase regulator (mxr) [Bb]
BB690	neutrophil activating prt (napA) [Hi]	57	BB020 pyroP-fructose 6-P 1-PPTase, sub (pfpB) [Bb]	100	BB416	pheromone shutdown prt (traB) [Ef]
BB179	thiophene and furan oxidation prt (thdF) [Bb]	100	BB055 trioseP isomerase [Bb]	100	BB042	P transport system regulatory prt (phoU) [Pa]
						57
<i>Protein and peptide secretion</i>			<i>Pentose phosphate pathway</i>		BB379	prt Kase C1 inhibitor (pkcl) [Bb]
BB154	preprt translocase sub (secA) [Bb]	100	BB222 glucose-6-P 1-DHase, put [As]	48	BB419	response regulatory prt (rrp-1) [Syn]
BB395	preprt translocase sub (secE) [Bl]	62	BB636 glucose-6-P 1-DHase (zwf) [Hi]	64		57
BB498	preprt translocase sub (secY) [Sc]	64	BB561 phosphogluconate DHase (gnd) [Sd]	71	BB763	response regulatory prt (rrp-2) [Ec]
BB362	prolipopt diacylglycerol Tase (lgt) [Ec]	56	BB657 ribose 5-P isomerase (rpi) [Mj]	61		67
BB652	prt-export membrane prt (secD) [Ec]	63	<i>Sugars</i>		BB764	sensory transduction histidine Kase, put [Bs]
BB653	prt-export membrane prt (secF) [Hi]	63	BB407 mannose-6-P isomerase (manA) [Ec]	54	BB420	sensory transduction histidine Kase, put [Syn]
BB030	signal peptidase I (lepB-1) [Bs]	51	BB444 nucleotide sugar epimerase [Vc]	69	BB693	xylose operon regulatory prt (xylR-1) [Th.]
BB031	signal peptidase I (lepB-2) [Syn]	57	BB676 phosphoglycolate PPase (gph) [Hi]	50		48
BB263	signal peptidase I (lepB-3) [St]	57	BB207 UTP-glucose-1-P uridylylTase (gtaB) [Bs]	63	BB831	xylose operon regulatory prt (xylR-2) [Syn]
BB469	signal peptidase II (lsp) [Sc]	60	BB545 xylulokinase (xylB) [Bs]	43		51
BB694	signal recognition particle prt (ffh) [Bs]	70			lp54	
BB610	trigger factor (tig) [Hi]	50			BBA07	chpAl prt, put [Ec]
			Fatty acid and phospholipid metabolism			55
<i>Transformation</i>			<i>General</i>		Replication	
BB591	competence locus E, put [Bs]	54	BB037 1-acyl-sn-glycerol-3-P AcTase (plsC) [Bb]	100	<i>Degradation of DNA</i>	
BB798	competence prt F, put [Hi]	52	BB685 3-OH-3-methylglutaryl-CoA RDase (mvaA) [Pm]	52	BB411	endonuclease precursor (nucA) [As]
			BB683 3-OH-3-methylglutaryl-CoA Sase (At)	53		53
Central intermediary metabolism			BB109 Ac-CoA C-AcTase (fadA) [Hi]	67	<i>DNA replication, restriction, modification, recombination, and repair</i>	
<i>General</i>			BB704 acyl carrier prt [Syn]	65	BB422	3-methyladenine DNA glycosylase (mag) [At]
BB241	glycerol kinase (glpK) [Ec]	74	BB721 CDP-diacylglycerol-glycerol-3-P 3-phosphatidylTase [Bs]	55	BB827	ATP-dep helicase (hrpA) [Ec]
BB243	glycerol-3-P DHase, anaerobic (glpA) [Hi]	52	BB327 glycerol-3-P O-acylTase, put [So]	50	BB437	chromosomal replication init prt (dnaA) [Bb]
BB376	SAM Sase (metK) [Bs]	72	BB368 glycerol-3-P DHase, NAD(P) ⁺ (gpsA) [Bs]	54	BB435	DNA gyrase, sub A (gyrA) [Bs]
<i>Amino sugars</i>			BB137 long-chain-fatty-acid CoA ligase (Syn)	54	BB436	DNA gyrase, sub B (gyrB) [Bb]
BB152	glucosamine-6-P isomerase (nagB) [Hi]	79	BB593 long-chain-fatty-acid CoA ligase [Syn]	56	BB344	DNA helicase (uvrD) [Ec]
BB151	N-Acglucosamine-6-P deAcase (nagA) [Hi]	54	BB688 mevalonate Kase [Mj]	51	BB552	DNA ligase (lig) [Ta]
			BB686 mevalonate pyroP DCase [Sc]	52	BB211	DNA mismatch repair prt (mutL) [Hi]
<i>Degradation of polysaccharides</i>			BB119 phosphatidate cytidylTase (cdsA), AFS[Ec]	61	BB797	DNA mismatch repair prt (mutS) [Hi]
BB620	-glucosidase, put [Syn]	58	BB249 phosphatidylTase [Hp]	52	BB098	DNA mismatch repair prt, put [Syn]
BB002	-N-Achexosaminidase, put [As]	54	BB687 phosphomevalonate Kase, put [Sc]	53		51
<i>Phosphorus compounds</i>					BB548	DNA polymerase I (polA) [Hi]
BB533	phnP prt (phnP) [Ec]	48			BB579	DNA polymerase III, sub (dnaE) [Ec]
<i>Polysaccharides - (cytoplasmic)</i>			Purines, pyrimidines, nucleosides, nucleotides		BB438	DNA polymerase III, sub (dnaN) [Bb]
BB166	4- -glucanase (malG) [Syn]	55	<i>Nucleotide and nucleoside interconversion</i>			100
BB004	phosphoglucomutase (femD) [Mj]	52	BB417 adenylate kinase (adk) [Bs]	64	BB461	DNA polymerase III, sub / (dnaX) [Bs]
BB835	phosphomannomutase (cpsG) [Hi]	57	BB128 cytidylate kinase (cmk-1) [Bs]	58	BB710	DNA primase (dnaG) [Bs]
			BB819 cytidylate kinase (cmk-2) [Mj]	57	BB581	DNA recombinase (recG) [Syn]
Energy metabolism			BB463 nucleoside-diP kinase (ndk) [Bs]	70	BB828	DNA topoisomerase I (topA) [Syn]
<i>Aerobic</i>			BB793 thymidylate kinase (tmk) [Mj]	59	BB035	DNA topoisomerase IV (parC) [Bb]
BB728	NADH oxidase, water-forming (nox) [Sh]	59	BB571 uridylate kinase (smbA) [Mj]	54	BB036	DNA topoisomerase IV (parE) [Bb]
					BB745	endonuclease III (nth) [Syn]
<i>Amino acids and amines</i>			<i>Purine ribonucleotide biosynthesis</i>		BB837	excinuclease ABC, sub A (uvrA) [Ec]
BB841	arginine deiminase (arcA) [Cp]	75	BB544 phosphoribosyl pyroP Sase (prs) [Mp]	59	BB836	excinuclease ABC, sub B (uvrB) [Ec]
BB842	ornithine carbamoylTase (arcB) [Ng]	74				71
			cp26		BB457	excinuclease ABC, sub C (uvrC) [Syn]
<i>Anaerobic</i>			BBB18 GMP Sase (guaA) [Bb]	100		57
BB016	glpE prt (glpE) [Hi]	53	BBB17 IMP DHase (guaB) [Bb]	100	BB534	exodeoxyribonuclease III (exoA) [Bs]
BB087	L-lactate DHase (ldh) [Bs]	72	<i>Pyrimidine ribonucleotide biosynthesis</i>		BB632	exodeoxyribonuclease V, chain

BB633	(recD) {Ec}	54	BB833	isoleucyl-tRNA Sase (ileS) {Sc}	66	BB703	ribosomal prt L32 (rpmF) {Bs}	62
	exodeoxyribonuclease V, chain (recB) {Hi}	51	BB251	leucyl-tRNA Sase (leuS) {Bs}	70	BB396	ribosomal prt L33 (rpmG) {Bs}	76
BB634	exodeoxyribonuclease V, chain (recC) {Hi}	51	BB659	lysyl-tRNA Sase {Mj}	54	BB440	ribosomal prt L34 (rpmH) {Bb}	100
BB829	exonuclease SbcD (sbcD) {Ec}	55	BB587	methionyl-tRNA Sase (metG) {Sc}	67	BB189	ribosomal prt L35 (rpmI) {Ba}	74
BB830	exonuclease SbcC (sbcC) {Ec}	52	BB514	phenylalanyl-tRNA Sase, sub (pheT) {Bb}	100	BB499	ribosomal prt L36 (rpmJ) {Bs}	89
BB177	glucose-inhibited div prt B (gidB) {Bb}	99	BB513	phenylalanyl-tRNA Sase, sub (pheS) {Bb}	100	BB127	ribosomal prt S1 (rpsA) {Ec}	55
BB178	glucose-inhibited div prt A (gidA) {Bb}	100	BB402	prolyl-tRNA Sase (proS) {Sc}	65	BB123	ribosomal prt S2 (rpsB) {Pa}	79
BB022	Holliday junction DNA helicase (ruvB) {Bb}	100	BB226	seryl-tRNA Sase (serS) {Bs}	62	BB484	ribosomal prt S3 (rpsC) {Hi}	71
BB023	Holliday junction DNA helicase (ruvA) {Bb}	100	BB720	threonyl-tRNA Sase (thrZ) {Bs}	67	BB615	ribosomal prt S4 (rpsD) {Hi}	63
BB014	primosomal prt N (priA) {Bb}	100	BB005	tryptophanyl-tRNA Sase (trsA) {Cl}	65	BB495	ribosomal prt S5 (rpsE) {Bs}	77
BB131	recA prt (recA) {Bb}	100	BB370	tyrosyl-tRNA Sase (tyrS) {Bs}	62	BB115	ribosomal prt S6 (rpsF) {Os}	50
BB607	rep helicase, ss DNA-dep ATPase (rep) {Hi}	61	BB738	valyl-tRNA Sase (valS) {Bs}	67	BB386	ribosomal prt S7 (rpsG) {Sc}	75
BB111	replicative DNA helicase (dnaB) {Ec}	58	<i>Degradation of proteins, peptides, and glycopeptides</i>			BB492	ribosomal prt S8 (rpsH) {Syn}	66
BB114	ss DNA-BP (ssb) {Syn}	62	BB608	aminoacyl-histidine dipeptidase (pepD) {Hi}	55	BB338	ribosomal prt S9 (rpsI) {Hi}	71
BB254	ss-DNA-specific exonuclease (recJ) {Hi}	52	BB366	aminopeptidase I (yscI) {Bb}	100	BB477	ribosomal prt S10 (rpsJ) {Bb}	100
BB623	transcription-repair coupling factor (mfd) {Hi}	60	BB069	aminopeptidase II {Bs}	57	BB501	ribosomal prt S11 (rpsK) {Hi}	77
BB053	uracil DNA glycosylase (ung) {Hi}	68	BB611	ATP-dep Clp protease proteolytic component (clpP-1) {Hi}	79	BB387	ribosomal prt S12 (rpsL) {An}	89
BBG32	replicative DNA helicase, put {Bs}	59	BB757	ATP-dep Clp protease proteolytic component (clpP-2) {Hi}	67	BB500	ribosomal prt S13 (rpsM) {Op}	76
BBE29	adenine specific DNA MTase, put {Hp}	57	BB369	ATP-dep Clp protease, sub A (clpA) {Ec}	56	BB491	ribosomal prt S14 (rpsN) {Bs}	72
Transcription			BB612	ATP-dep Clp protease, sub X (clpX) {Ec}	75	BB804	ribosomal prt S15 (rpsO) {Ti}	77
<i>General</i>			BB834	ATP-dep Clp protease, sub C (clpC) {Pp}	67	BB695	ribosomal prt S16 (rpsP) {Bs}	70
BB052	spoU prt (spoU) {Ec}	54	BB253	ATP-dep protease LA (lon-1) {Bb}	100	BB487	ribosomal prt S17 (rpsQ) {Mc}	76
<i>Degradation of RNA</i>			BB613	ATP-dep protease LA (lon-2) {Hi}	65	BB113	ribosomal prt S18 (rpsR) {Bs}	78
BB805	polyribonucleotide nucleotidylTase (pnpA) {Bs}	68	BB359	carboxyl-terminal protease (ctp) {Syn}	65	BB482	ribosomal prt S19 (rpsS) {Bb}	99
BB046	ribonuclease H (rnhB) {Hi}	66	BB203	Lambda CII stability-governing prt (hflK) {Ec}	56	BB233	ribosomal prt S20 (rpsT) {Bb}	100
BB705	ribonuclease III (rnc) {Bs}	62	BB204	Lambda CII stability-governing prt (hflC) {Ec}	56	BB256	ribosomal prt S21 (rpsU) {Mx}	68
BB441	ribonuclease P prt component (rnpA) {Bb}	100	BB248	oligoendopeptidase F (pepF) {Li}	58	BB516	rRNA methylase (yacO) {Mc}	66
<i>DNA-dependent RNA polymerase</i>			BB067	peptidase, put {Sc}	56	<i>tRNA modification</i>		
BB502	DNA-directed RNA polymerase (rpoA) {Bs}	64	BB104	periplasmic serine protease DO (htrA) {Hi}	60	BB821	2-methylthio-N6-isopentyladenosine tRNA modification enzyme (miaA) {Ec}	53
BB389	DNA-directed RNA polymerase (rpoB) {Bb}	97	BB430	proline dipeptidase (pepQ) {Hi}	49	BB084	AT (nifS) {Syn}	61
BB388	DNA-directed RNA polymerase (rpoC) {Ec}	71	BB769	sialoglycoprotease (gcp) {Hi}	60	BB343	glu-tRNA amidotase, sub C (gatC) {Bs}	56
BB771	RNA polymerase sigma factor (rpoS) {Pa}	61	BB627	vacuolar X-prolyl dipeptidyl aminopeptidase I (pepX) {Mi}	55	BB341	glu-tRNA amidotase, sub B (gatB) {Bs}	63
BB712	RNA polymerase sigma-70 factor (rpoD) {Bb}	100	BB118	zinc protease, put {Hi}	54	BB342	glu-tRNA amidotase, sub A (gatA) {Bs}	61
BB450	RNA polymerase sigma-54 factor (ntrA) {Av}	57	BB536	zinc protease, put {Hi}	52	BB064	methionyl-tRNA formylTase (fmt) {Ec}	56
<i>Transcription factors</i>			<i>Nucleoproteins</i>			BB787	peptidyl-tRNA hydrolase (pth) {Bb}	100
BB107	N utilization substance prt B (nusB) {Ec}	62	BB232	hbbU prt {Bb}	100	BB012	pseudouridylylase I (hisT) {Bb}	100
BB800	N-utilization substance prt A (nusA) {Bs}	62	<i>Protein modification</i>			BB021	SAM: tRNA ribosylTase-isomerase {Bb}	96
BB394	transcription antitermination factor (nusG) {Ec}	64	BB105	methionine aminopeptidase (map) {Bs}	68	BB809	tRNA-guanine transglycosylase (tgt) {Zm}	60
BB132	transcription elongation factor (greA) {Ec}	56	BB065	polypeptide deformylase (def) {Syn}	67	BB698	tRNA (guanine-N1)-MTase (trmD) {Mg}	68
BB355	transcription factor, put {Mx}	47	BB648	serine/threonine kinase, put {Pf}	51	BB803	tRNA pseudouridine 55 Sase (truB) {Ec}	57
BB230	transcription termination factor Rho (rho) {Bb}	100	<i>Ribosomal proteins: synthesis and modification</i>			<i>Translation factors</i>		
<i>RNA processing</i>			BB392	ribosomal prt L1 (rplA) {Bs}	71	BB088	GTP-B membrane prt (lepA) {Hi}	76
BB706	polynucleotide adenylTase (papS) {Bs}	57	BB481	ribosomal prt L2 (rplB) {Bb}	99	BB196	peptide chain release factor 1 (prfA) {Hi}	73
Translation			BB478	ribosomal prt L3 (rplC) {Bb}	99	BB074	peptide chain release factor 2 (prfB) {Sc}	70
<i>General</i>			BB479	ribosomal prt L4 (rplD) {Bb}	100	BB121	ribosome releasing factor (frr) {Mt}	68
BB590	dimethyladenosine Tase (ksgA) {Bs}	61	BB490	ribosomal prt L5 (rplE) {Hi}	80	BB169	translation initiation factor 1 (infA) {Ec}	87
BB802	ribosome-B factor A (rbfA) {Bs}	62	BB493	ribosomal prt L6 (rplF) {Sc}	72	BB801	translation initiation factor 2 (infB) {Bs}	73
<i>Amino acyl tRNA synthetases</i>			BB390	ribosomal prt L7/L12 (rplL) {Sc}	75	BB190	translation initiation factor 3 (infC) {Pv}	72
BB220	alanyl-tRNA Sase (alaS) {Ec}	62	BB112	ribosomal prt L9 (rplI) {Ec}	57	BB691	translation elongation factor G (fus-2) {Tm}	67
BB594	arginyl-tRNA Sase (argS) {Mj}	55	BB391	ribosomal prt L10 (rplJ) {Bs}	61	BB214	translation elongation factor P (efp) {Ec}	56
BB101	asparaginyl-tRNA Sase (asnS) {Ec}	73	BB393	ribosomal prt L11 (rplK) {Tm}	73	BB476	translation elongation factor TU (tuf) {Bb}	100
BB446	aspartyl-tRNA Sase (aspS) {Ec}	66	BB339	ribosomal prt L13 (rplM) {Hi}	72	BB122	translation elongation factor TS (tsf) {Hi}	57
BB599	cysteinyl-tRNA Sase (cysS) {Hi}	58	BB488	ribosomal prt L14 (rplN) {Tm}	79	BB540	translation elongation factor G (fus-1) {Tm}	68
BB372	glutamyl-tRNA Sase (gluX) {Rm}	63	BB497	ribosomal prt L15 (rplO) {Bs}	68	Transport and binding proteins		
BB371	glycyl-tRNA Sase (glyS) {Ta}	68	BB485	ribosomal prt L16 (rplP) {Syn}	81	<i>General</i>		
BB135	histidyl-tRNA Sase (hisS) {Mj}	59	BB503	ribosomal prt L17 (rplQ) {Ec}	63	BB573	ABC transporter, ATP-BP {Bs}	53
			BB494	ribosomal prt L18 (rplR) {Bs}	69	BB742	ABC transporter, ATP-BP {Syn}	57
			BB699	ribosomal prt L19 (rplS) {Ec}	74	BB466	ABC transporter, ATP-BP {Hi}	74
			BB188	ribosomal prt L20 (rplT) {Ec}	70	BB754	ABC transporter, ATP-BP {Bi}	60
			BB778	ribosomal prt L21 (rplU) {Ec}	58	BB080	ABC transporter, ATP-BP {Mj}	63
			BB483	ribosomal prt L22 (rplV) {Bb}	100	BB269	ATP-BP (yixH-1) {Bb}	100
			BB480	ribosomal prt L23 (rplW) {Bb}	100	BB726	ATP-BP (yixH-2) {Bb}	54
			BB489	ribosomal prt L24 (rplX) {Ec}	64	<i>Amino acids, peptide, and amines</i>		
			BB780	ribosomal prt L27 (rpmA) {Hi}	82	BB729	glutamate transporter (gluTP) {Bs}	55
			BB350	ribosomal prt L28 (rpmB) {Ec}	62	BB401	glutamate transporter, put {Bs}	53
			BB486	ribosomal prt L29 (rpmC) {Bs}	65	BB146	GBP ABC transporter, ATP-BP	
			BB496	ribosomal prt L30 (rpmD) {Bs}	60			
			BB229	ribosomal prt L31 (rpmE) {Bs}	69			

	(proV) {Sc}	71		{Mg}	56	BB586	femA prt (femA) {Se}	47
BB145	GBP ABC transporter, permease prt (proW) {Ec}	66	BB557	phosphocarrier prt HPr (ptsH-2) {Hi}	69	BB141	membrane fusion prt (mtrC) {Hi}	47
BB144	GBP ABC transporter, BP (proX) {Ec}	43	BB558	phosphoenolpyruvate-PT PPase (ptsI) {Sc}	65	<u>lp28-4</u>	multidrug-efflux transporter {Hp}	55
BB334	OP ABC transporter, ATP-BP (oppD) {Bs}	75	BB408	PTS system, fru-specific IIBC (fruA-1) {Ec}	65	<u>lp25</u>		
BB335	OP ABC transporter, ATP-BP (oppF) {Bs}	80	BB629	PTS system, fru-specific IIBC (fruA-2) {Ec}	68	BBE22	pyrazinamidase/nicotinamidase (pncA) {Mt}	56
BB332	OP ABC transporter, permease prt (oppB-1){Ec}	68	BB559	PTS system, glu-specific IIA (crr) {Bb}	100	<i>Transposon-related functions</i>		
BB747	OP ABC transporter, permease prt (oppB-2){Bs}	54	BB645	PTS system, glu-specific IIBC (ptsG) {Sc}	67	<u>lp38</u>		
BB333	OP ABC transporter, permease prt (oppC-1){Hi}	64	BB116	PTS system, mal/glu-specific IIBC (malX) {Ec}	56	BBJ05	transposase-like prt, put {Bb}	89
BB746	OP ABC transporter, permease prt (oppC-2){Bs}	52	BB677	RG ABC transporter, ATP-BP (mgIA) {Mg}	68	BBK25	transposase-like prt, put {Bb}	80
BB328	OP ABC transporter, periplasmic BP (oppA-1) {Bb}	74	BB678	RG ABC transporter, permease prt (rbsC-1) {Mg}	51	<u>lp28-1</u>		
BB329	OP ABC transporter, periplasmic BP (oppA-2) {Bb}	94	BB679	RG ABC transporter, permease prt (rbsC-2) {Mp}	52	BBF18	transposase-like prt, put {Bb}	96
BB330	OP ABC transporter, periplasmic BP (oppA-3) {Bb}	81	<u>cp26</u>			BBF19	transposase-like prt, put {Bb}	96
BB642	SP ABC transporter, ATP-BP (potA) {Ec}	69	BBB04	PTS system, cello-specific IIC (celB) {Bs}	62	<u>lp28-2</u>		
BB641	SP ABC transporter, permease prt (potB) {Ec}	65	BBB05	PTS system, cello-specific IIA (celC) {Bs}	61	BBG05	transposase-like prt {Bb}	99
BB640	SP ABC transporter, permease prt (potC) {Ec}	63	BBB06	PTS system, cello-specific IIB (celA) {Bs}	73	<u>lp28-3</u>		
BB639	SP ABC transporter, periplasmic BP (potD) {Ec}	53	BBB29	PTS system, glu-specific IIBC, put {Ec}	70	BBH40	transposase-like prt, put {Bb}	57
<u>lp54</u>			<i>Cations</i>			<u>lp17</u>		
BBA34	OP ABC transporter, periplasmic BP (oppA-4) {Bc}	66	BB724	K+ transport prt (ntpJ) {Eh}	60	BBD20	transposase-like prt, put {Bb}	99
<u>cp26</u>			BB380	Mg2+ transport prt (mgtE) {Bb}	100	BBD23	transposase-like prt, put {Bb}	88
BBB16	OP ABC transporter, periplasmic BP (oppA) {Bb}	78	BB164	Na+/Ca+ exchange prt, put {Mj}	59	Unknown		
<i>Anions</i>			BB447	Na+/H+ antiporter (napA) {Eh}	57	BB528	aldose RDase, put {Bs}	57
BB218	P ABC transporter, ATP-BP (pstB) {Pa}	74	BB637	Na+/H+ antiporter (nhaC-1) {Bf}	48	BB684	carotenoid biosyn prt, put {Ss}	58
BB216	P ABC transporter, permease prt (pstC) {Ec}	58	BB638	Na+/H+ antiporter (nhaC-2) {Hi}	50	BB671	chemotaxis operon prt (cheX) {Bb}	99
BB217	P ABC transporter, permease prt (pstA) {Syn}	63	<i>Other</i>			BB250	dedA prt (dedA) {Ec}	54
BB215	P ABC transporter, periplasmic P-BP (pstS) {Syn}	48	BB451	chromate transport prt, put {Mj}	58	BB168	dnaK suppressor, put {Ec}	53
<i>Carbohydrates, organic alcohols, and acids</i>			Other categories			BB508	GTP-BP {Tp}	59
BB240	glycerol uptake facilitator (glpF) {Bs}	57	<i>Adaptations and atypical conditions</i>			BB219	gufA prt {Mx}	54
BB604	L-lactate permease (lctP) {Ec}	57	BB237	acid-inducible prt (act206) {Rm}	45	BB421	hydrolase {Hi}	58
BB318	methylgalactoside ABC transporter, ATP-BP (mgIA) {Hi}	54	BB786	general stress prt (ctc) {Bs}	51	BB524	inositol monoPPase {Hs}	47
BB814	pantothenate permease (panF) {Ec}	63	BB785	stage V sporulation prt G {Bm}	74	BB454	lipopolysaccharide biosyn-related prt {Mj}	49
BB448	phosphocarrier prt HPr (ptsH-1)		BB810	virulence factor mviN prt (mviN) {Hi}	51	BB702	lipopolysaccharide biosyn-related prt {Hi}	62
			<i>Colicin-related functions</i>			BB045	P115 prt {Mh}	53
			BB766	colicin V production prt, put {Hi}	52	BB336	P26 {Bb}	100
			BB546	outer membrane integrity prt (tolA) {Hi}	44	BB363	periplasmic prt {Bb}	100
			<i>Drug and analog sensitivity</i>			BB033	small prt (smpB) {Rp}	70
			BB140	acriflavine resistance prt (acrB) {Hi}	53	BB297	smg prt {Bb}	100
			BB258	bacitracin resistance prt (bacA) {Ec}	56	BB443	spolIJ-associated prt (jag) {Bs}	56
						<u>lp54</u>		
						BBA76	thy1 prt (thy1) {Dd}	68
						<u>lp28-4</u>		
						BBI06	pfs prt (pfs) {Ec}	59
						<u>cp9</u>		
						BB009	rev prt (rev) {Bb}	62
						BB010	rev prt (rev) {Bb}	66

Increasing X-ray emissions and periodic outbursts from the massive star η Carinae

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Eta Carinae is one of the most massive, luminous and unstable stars known. The basic nature of this star is poorly understood, despite much study^{1,2}. It is a fluctuating source of hard X-rays^{3–5}, indicative of gas at an unusually high temperature (60 million kelvin): the mechanism for producing this gas has yet to be established, but may be related to strong shocks in a dense stellar wind. We have monitored the hard X-ray emission (2–10 keV) from η Car over the past 1.5 years, in order to better understand the nature of these emissions and their variations. We show here that there has been an overall increase in the mean X-ray flux which has accelerated since January 1997, and that there are also small-scale periodic outbursts that occur every 85 days. It has recently been argued^{6,7} that η Car is in fact a binary stellar system whose components are approaching periastron near 1 January 1998. If this is indeed the case, then it is plausible that the hard X-ray emission is produced by shocks associated with the collision of the winds from the two stars, in which case the X-ray flux should increase through periastron, and rapidly decline thereafter. Continued monitoring will test this prediction.

The X-ray variability of this source is illustrated by two false-colour X-ray images (Fig. 1, left and centre), obtained in August 1992 and July 1994. An optical image⁸ obtained by the Wide Field/Planetary Camera (WFPC2) on the Hubble Space Telescope is shown (Fig. 1, right) for comparison. The extensive optical nebulosity (often called “the homunculus”⁹) in the WFPC2 image was

ejected in a giant outburst seen 150 years ago, and is surrounded by a cocoon of X-ray-emitting gas at a temperature of $\sim 2 \times 10^6$ K (refs 4, 5). An unresolved ‘knot’ or ‘core’ of emission coincident with the optical star has a much harder thermal spectrum and must be produced in an extremely hot plasma close to the star. The X-ray emission from this unresolved source varies³, as can be seen in Fig. 1: it was invisible in 1992 but clearly visible in 1994. The gas at 60×10^6 K may be produced by strong shocks located somewhere near the star^{4,5}. The variability in the hard emission on timescales of months³ suggests fairly rapid evolution in the observed hot gas. In order to better define the X-ray variability timescale, the hard X-ray emission from η Car has been monitored approximately once per week on average since February 1996 by the Proportional Counter Array (PCA) on board the RXTE satellite¹⁰. The PCA consists of five collimated proportional counters sensitive to X-ray photons in the energy range 2–60 keV.

Figure 2 (top panel) shows the PCA X-ray ‘lightcurve’ in the 2–10 keV band corrected for internal and cosmic background. This represents the first detailed portrait of the temporal behaviour of the X-ray flux from η Car. The net X-ray emission shows a progressive brightening throughout the monitoring interval, with shorter variations about this mean increase. Before January 1997 the average rate of increase was 0.005 PCA counts $s^{-1} d^{-1}$; after January 1997 the rate of increase accelerated to 0.066 PCA counts $s^{-1} d^{-1}$. The conversion between PCA rates and 2–10 keV flux is $\sim 3.5 \times 10^{-12}$ erg cm^{-2} count $^{-1}$.

Variability on timescales of days–weeks is characterized by recurrent peaks or ‘outbursts’ in which the observed count rate rises by 10–30% for brief periods. A formal statistical estimate of the average interval between peaks, based on all observed outburst events, is 84.8 ± 1.2 d; the r.m.s. deviation for a single event is ~ 5 d. We noticed this periodicity in April 1997 and used it to predict the next peak¹¹. Data from the interval around the predicted peak are shown in Fig. 2 inset. The X-ray count rate peaked on JD 2,450,635.09 $\pm$ 1.10 (UT 5 July 1997 13:54), very near the time of the predicted ‘outburst’¹².

Because the PCA detects X-ray emission from a 1° (full-width at half-maximum, FWHM) region around η Car, there is the possibility that emission from another X-ray variable in the field could be mistaken for variability from η Car. At the location of η Car, cosmic background emission in the 2–10 keV energy range is thought to be dominated by non-varying diffuse emission from the Galactic Ridge¹³. Other known X-ray point sources near η Car are much weaker than that star and produce very little emission at energies

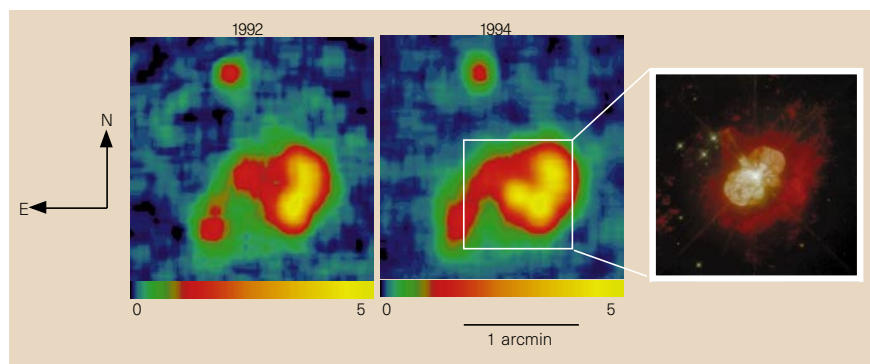


Figure 1 False-colour X-ray images of η Car and a Hubble Space Telescope optical image. The X-ray images (left, centre) were obtained using the High Resolution Imager (HRI) on the *Röntgensatellit* (Rosat) X-ray observatory²³ during pointed-phase observations in 1992 and 1994. The HRI detects X-ray photons in the 0.2–2.4 keV energy range and records their spatial distribution with a precision of a few arcseconds. The X-ray images shown here were constructed from all photons which passed the standard acceptance criteria. The X-ray emission

around η Car arises from extended, relatively soft emission surrounding a point-like, hard, source. The point-like source is variable; it was nearly invisible in 1992 but much brighter in 1994. The colour scales give the number of HRI counts per pixel per 10,000 s. For comparison the optical image of η Car and the ‘homunculus’ (obtained with the Hubble Space Telescope WFPC2 instrument; right) is also shown.

above 2 keV (refs 4, 5). Nevertheless, the possibility exists that an unknown variable source in the field could contaminate the 2–10 keV emission detected by the PCA. In particular, a weak X-ray source, HD92740, is a spectroscopic binary with an orbital period of 80.3 d (ref. 14), and is located only 28 arcmin from η Car. The orbital period of HD92740 is suspiciously close to the period of the outbursts in our PCA 'lightcurve'. As a check, we phased the timings of X-ray maxima with the 80.3-d orbital period of HD92740. This showed that the X-ray maxima drift in phase with respect to the orbit of HD92740, making it unlikely that HD92740 is the source of the X-ray variability. To further verify that the variations we detect in the PCA observations are indeed produced by η Car, and not HD92740 or some other object, we obtained spatially resolved spectra of η Car on 3 and 19 July 1997 with the ASCA X-ray satellite observatory¹⁵. The ASCA observatory consists of a thin-foil X-ray mirror providing arcminute spatial resolution in the energy range 0.5–10 keV, along with two sets of X-ray imaging detectors in the focal plane. The ASCA data thus provide a direct measure of the X-ray flux of η Car that is free of contamination from any significant source in the field (including HD92740). The ASCA spectrum of η Car obtained on 3 July, near the peak of the outburst, is $\sim 12\%$ brighter in the 2–10 keV band than the spectrum obtained on 19 July. This is in reasonable agreement with the RXTE data obtained on these dates, and confirms beyond doubt that η Car is in fact the source of the variations we report.

The rapid rise and fall of the X-ray emission during the outbursts suggests sudden heating and cooling of the plasma. If radiative cooling dominates, then the cooling time is $\tau_{\text{rad}} = 8.6 \times 10^{10} n_e^{-1} T^{1/2}$ seconds, with n_e the electron number density and T

the temperature in K. We estimate the cooling rate from the time it takes the X-ray emission to reach 1/e of the peak value; this is ~ 3 –22 d for all the observed peaks. For a plasma with $T \approx 60 \times 10^6$ K, and taking $\tau_{\text{rad}} \approx 15$ d, the inferred electron density is about $n_e \approx 5.3 \times 10^8 \text{ cm}^{-3}$. If this density is representative of the density in the wind from η Car at the location of the hot plasma, then by conservation of mass the distance from the star to the hot plasma is $D \approx 1.8 \times 10^{14} (\dot{M}_{-4}/(v_5 n_{e,8}))^{1/2} \text{ cm}$, where $\dot{M}_{-4}$ is the mass loss rate in units of $10^{-4} M_{\odot} \text{ yr}^{-1}$ ($M_{\odot}$ is the solar mass), v_5 the wind speed in units of 500 km s⁻¹, and $n_{e,8}$ the electron density in units of 10^8 cm^{-3} . Recent estimates¹⁶ suggest $v_5 \approx 1$ and $\dot{M}_{-4} = 1$, so $D \approx 8 \times 10^{13} \text{ cm}$. This is about ~ 100 times closer to the star than the previous best estimate⁴. An earlier suggestion⁴ that the hard emission is produced by the collision of η Car's stellar wind with distant dust clouds now seems untenable, as the X-ray region appears to be well inside the dust-forming zone.

What does the 85-d period represent? Given the likely physical parameters of η Car (refs 1, 2), three obvious possibilities all seem consistent with this timescale. (1) It may be the orbital period of a close binary system. With $P = 85$ d, and a total mass of the order of $120 M_{\odot}$, the average separation between components would be $\sim 3 \times 10^{13} \text{ cm}$. X-ray variability modulated on the orbital timescale could conceivably result from the collision¹⁷ of the winds from the two component stars. (2) The period could represent the stellar rotation period. One speculative model for the X-ray outbursts in this case might involve streams of fast-moving material from the star colliding with localized slow-moving gas once per rotation period, similar to the "corotating interaction regions"¹⁸ which may exist in the winds of other massive stars. (3) The X-ray period could represent a stellar pulsation period. Ordinarily the dynamical timescale of a star with this mass and size would be < 85 d, but η Car is only marginally stable and that increases the characteristic period. A photospheric pulsation could modulate the density in the stellar wind, which could manifest itself as a periodic variation in the volume emission measure (and resultant flux) of the X-ray zone. Other models are of course possible.

Though no 85-d optical periodicity has been noticed in η Car, a weak photometric effect with a period of ~ 58 d has been suspected¹⁹. It seems interesting to us that 85 and 58 d are close to a 3:2 ratio. When a small-integer commensurability occurs in astrophysics, it usually involves either a pair of orbits, or an orbital period and a rotation period. (A famous example is the planet Mercury, with its 88-d orbit and 59-d rotation.) This may suggest the presence of additional gravitationally bound massive bodies in the system.

Whatever their origins, the η Car X-ray variations are unique among massive stars. The 'outburst' activity suggests small, fairly rapid changes in the thermodynamic state of the 60×10^6 K plasma. On the other hand, the acceleration of the increase in the mean X-ray flux suggests that the entire emitting region is perhaps moving toward some drastic change or 'crisis' during which the fundamental properties of the emission zone change suddenly. Although we do not have any firm indication of the nature of this 'crisis', we point out that the only types of massive stars known to show large, long-term variations in X-ray emission are 'colliding-wind' binaries. In these systems X-ray emission is generated at the shocked boundary where the winds from the stars collide. Theoretical models²⁰ and observations^{21,22} of a sample of eccentric, massive binaries show large, progressive increases in X-ray emission (similar to those seen in η Car) as the stars approach periastron, after which the emission decreases rapidly. More to the point, recent evidence^{6,7} suggests that in fact η Car may be an eccentric binary system with a 5.5-yr orbital period whose components are currently approaching periastron, predicted to occur near 1 January 1998. If so, then the system would probably be the most massive binary known. If the 2–10 keV emission from η Car is in fact produced by colliding winds, then the X-ray flux should continue to brighten rapidly through

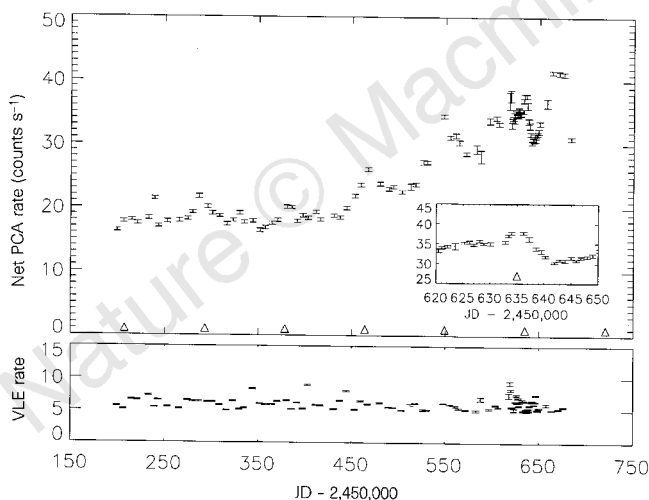


Figure 2 X-ray data from RXTE measurements of η Car. Top panel, the background-corrected RXTE PCA X-ray 'lightcurve' of η Car in the interval April 1996 to August 1997. The observed variations represent changes in X-ray flux in the 2–10 keV energy band. The times of the X-ray peaks are marked by triangles. The inset shows the results of daily monitoring near the predicted X-ray peaks in July 1997. The PCA consists of 5 collimated X-ray proportional counter units (PCUs) sensitive to X-ray photons in the energy range 2–60 keV. Each PCU views a 1° (FWHM) region of the sky centred on the source. For each observation, we extracted average count rates and spectra from PCUs 0, 1, 2 in the energy range 2–10 keV. We used only those observations made since the last change of the detector gain in April 1996, as these observations are better calibrated than earlier ones. The internal background was estimated for each observation assuming the particle flux is proportional to the rate of "Very Large Events" seen by the detectors. Bottom panel, the variation of the estimated detector background contribution. The derived PCA count rates have also been corrected for a sky background contribution of 18.71 ± 0.16 PCA counts s⁻¹ in all observations; the sky background was determined by comparison of PCA and ASCA spectra of η Car obtained in July 1996.

periastron passage, with a quick decline thereafter. The absorption suffered by the X-ray emission should show a similar rapid increase through periastron as the line of sight to the X-ray zone encounters increasingly larger amounts of stellar-wind material. We expect that continued monitoring of the time variations of X-ray brightness, absorbing column and temperature throughout 1998 should provide crucial evidence of the suspected binary nature of η Car. $\square$

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- Davidson, K. & Humphreys, R. M. Eta Carinae and its environment. *Annu. Rev. Astron. Astrophys.* **35**, 1–32 (1997).
- Davidson, K. in *Physics of Luminous Blue Variables* (eds Davidson, K., Moffat, A. & Lamers, H.) 101–107 (Kluwer, Dordrecht, 1989).
- Corcoran, M. F., Rawley, G. L., Swank, J. H. & Petre, R. First detection of X-ray variability from Eta Carinae. *Astrophys. J.* **445**, L121–L124 (1995).
- Chlebowski, T., Seward, F. D., Swank, J. H. & Szymkowiak, A. E. X-rays from Eta Carinae. *Astrophys. J.* **281**, 665–672 (1984).
- Corcoran, M. F. *et al.* The ASCA X-ray spectrum of η Carinae. *Astrophys. J.* (in the press).
- Damineli, A., Conti, P. S. & Lopes, D. F. Eta Carinae: a long period binary? *New Astron.* **2**, 387–390 (1997).
- Davidson, K. Is Eta Carinae a long-period binary? *New Astron.* **2**, 387–390 (1997).
- Morse, J. Examining Eta Carinae. *Sky Telesc.* **92**(4), 13 (1996).
- Gaviola, E. Eta Carinae. I. The nebulosity. *Astrophys. J.* **111**, 408–413 (1950).
- Bradt, H. V., Rothschild, R. E. & Swank, J. H. The X-ray timing explorer mission. *Astron. Astrophys. Suppl. Ser.* **97**, 355–360 (1993).
- Ishibashi, K. *et al.* Eta CARINAE. *IAU Circ.* No. 6668 (1997).
- Corcoran, M. F., Swank, J. H., Petre, R., Ishibashi, K. & Davidson, K. Eta CARINAE. *IAU Circ.* No. 6701 (1997).
- Koyama, K., Makishima, K., Tanaka, Y. & Tsunemi, H. Thermal X-ray emission with intense 6.7 keV iron line from the Galactic Ridge. *Publ. Astron. Soc. Jpn* **38**, 121–131 (1986).
- Rauw, G. *et al.* WR 22: the most massive Wolf-Rayet star ever weighed. *Astron. Astrophys.* **306**, 771–782 (1996).
- Tanaka, Y., Inoue, H. & Holt, S. S. The X-ray astronomy satellite ASCA. *Publ. Astron. Soc. Jpn* **46**, L37–L41 (1994).
- Hillier, J. D. & Allen, D. A. A spectroscopic investigation of Eta Carinae and the Homunculus Nebula. I—Overview of the spectra. *Astron. Astrophys.* **262**, 153–170 (1992).
- Prilutskii, O. F. & Usov, V. V. X-rays from Wolf-Rayet binaries. *Sov. Astron.* **20**, 2–3 (1976).
- Mullan, D. Displaced narrow absorption components in the spectra of mass-losing OB stars—Indications of corotating interaction regions? *Astron. Astrophys.* **165**, 157–162 (1986).
- Sterken, C., de Groot, M. J. H. & van Genderen, A. M. A pulsating star inside eta Carinae. II. The variability of the pulsation period. *Astron. Astrophys. Suppl. Ser.* **116**, 9–14 (1996).
- Stevens, I. R., Blondin, J. M. & Pollock, A. M. T. Colliding winds from early-type stars in binary systems. *Astrophys. J.* **386**, 265–287 (1992).
- Willis, A. J., Schild, H. & Stevens, I. R. ROSAT observations of γ Velorum (WC8+O9I). I. The discovery of colliding-wind X-ray emission. *Astron. Astrophys.* **298**, 549–566 (1995).
- Corcoran, M. F. X-ray emission from colliding wind binaries. *Rev. Mex. Astron. Astrophys. Conf. Ser.* **5**, 54–60 (1996).
- Trümper, J. The ROSAT mission. *Adv. Space Res.* **2**, 241–249 (1982).

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Controlling the sign of quantum interference by tunnelling from quantum wells

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The sign of the interference (constructive or destructive) between quantum-mechanical paths depends on the phase difference between the paths. In the Fano effect¹ two optical paths from the ground state of a system—one direct and one mediated by a resonance—to a state in an energy continuum interfere to produce an asymmetric absorption spectrum that falls to zero near the absorption maximum. Zero absorption occurs as the wavelength is scanned across the resonance, at a photon energy

corresponding to a 180° phase difference between the paths. Similar interference effects occur when two absorption paths are mediated by two different states, and they provide the basis for lasers that operate without a population inversion^{2–7}. Here we report the control, by quantum mechanical tunnelling, of interference in optical absorption. The two intermediate states are resonances that arise from the mixing of the states in two adjacent semiconductors quantum wells, which are broadened by tunnelling into the same energy continuum through an ultra-thin potential-energy barrier. Inverting the direction of tunnelling by reversing the position of the barrier with respect to the two quantum wells changes the interference from destructive to constructive, as predicted theoretically. This effect might provide a way to make semiconductor lasers without population inversion⁸.

The key features of our structures are a deep and a shallow well coupled by tunnelling to a single continuum of energies through a thin barrier (Fig. 1). The ground state of the shallow well and the first excited state of the deep well mix (that is, quantum mechanically anticross) to create two excited states ($|2\rangle$ and $|3\rangle$ in Fig. 1). Because the latter are strongly coupled to a continuum, they should be viewed as continuum resonances with a width dominated by the tunnelling probability through the thin barrier. These resonances can be represented, in a tight-binding approximation, as superpositions of the ground state and the first excited state of the isolated shallow and deep wells, respectively. The boundary condition on one side of the structure is set by a very thick (ideally infinite) barrier to suppress any tunnelling. This feature allows us to describe the wavefunctions with real numbers. Figure 1 shows that there are two ways of constructing such a quantum system, with either the shallow or the deep well adjacent to the thin barrier. Consider a state in the continuum at an energy in between the two resonances,

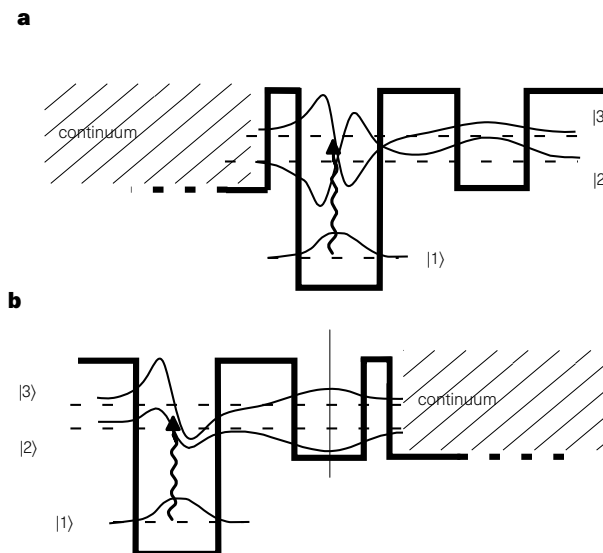


Figure 1 Schematic band diagram of the quantum well structures used to demonstrate quantum interference in optical absorption from the ground state $|1\rangle$ to the two continuum resonant states $|2\rangle$ and $|3\rangle$ which are predominantly broadened by tunnelling. The solid curves are schematics of the electronic wavefunctions at the ground-state and resonance energies. The wavy arrow represents the optical transition. **a**, When the thin tunnelling barrier is adjacent to the deep well, the wavefunction of the final state of the transition experiences a 180° phase shift as the energy of the incident photon is scanned between the two resonances. This causes a change of sign in the transition matrix element and therefore a zero in the absorption coefficient (destructive interference) at an intermediate energy. **b**, When the thin barrier is adjacent to the shallow well there is no 180° phase shift, so there is constructive interference.

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which have a significant overlap due to their large broadening. The probability amplitude for optical absorption from $|1\rangle$ to that state can be thought as the superposition of two paths, one via the lower and one via the upper resonance. We will show that the sign of the interference is opposite (destructive and constructive) in the two structures. We have chosen the arbitrary phase factor of the wavefunctions such that inside the infinitely thick barrier, they are positive for all energies. Consider the structure of Fig. 1a: as the photon energy is changed, the absorption matrix element changes sign as one moves from the lower to the upper resonance because the sign of the wavefunction in the deep well changes. The change of sign implies that at a certain energy the modulus of the matrix element vanishes and thus the absorption coefficient is zero (destructive interference). In the other structure (Fig. 1b), the matrix element does not change sign because the wavefunction in the deep well does not experience a phase shift; this leads to an enhancement of absorption (constructive interference).

For a simple analytical description of these phenomena, consider the normalized absorption cross-section σ_{abs} in a quantum system in which two upper states are coupled to the same continuum by tunnelling. In the limit of a negligible dipole matrix element from the ground state to the continuum, σ_{abs} reads²

$$\sigma_{\text{abs}} = \frac{(\eta_3 + \eta_2 q)^2}{\eta_2^2 \eta_3 + (\eta_2 + \eta_3)^2} \quad (1)$$

In this expression $\eta_3 = 2(E_{31} - h\nu)/\Gamma_3$ and $\eta_2 = 2(E_{21} - h\nu)/\Gamma_2$ are the normalized energy detunings, $h\nu$ is the photon energy, E_{31} and E_{21} are the transition energies between the $n = 1$ ground state and the two resonances, and Γ_3 and Γ_2 are their full-widths at half-maximum, proportional to the tunnelling rates into the continuum. It is assumed that other broadening associated with scattering is negligible compared with Γ_3 and Γ_2 . In the spirit of Fano's original

work¹, the parameter

$$q = \frac{z_{13}}{z_{12}} \sqrt{\frac{\Gamma_2}{\Gamma_3}} \quad (2)$$

where z_{12} and z_{13} are the matrix elements to levels E_2 and E_3 , respectively, controls the magnitude of the interference effect. As in Fano's work¹, the sign of the matrix element to the continuum, even though not explicit in equations (1) and (2), guarantees that the sign of q does not depend on the arbitrary phase factor of the wavefunctions. It is apparent from equation (1) that the sign of q controls the sign of the interference effect (constructive or destructive).

The grey-scale density plot of the squares of the wavefunctions for both structures, displayed in Fig. 2, provides additional physical insight into the origin of this asymmetry. If the tunnel barrier is placed on the side of the GaAs deep well (Fig. 2a), the resonance present in the AlGaAs shallow well provides the phase shift for the wavefunction in the deep well which causes the suppression of the absorption coefficient between the two resonances (solid lines in Fig. 3). On the contrary, if the tunnel barrier is placed on the side of the shallow well (Fig. 2b), the phase of the wavefunction in the deep well remains the same and the two absorption resonances interfere constructively (dashed lines in Fig. 3).

Our structures are grown by molecular-beam epitaxy on semi-insulating GaAs substrates. After a 1- μm -thick undoped GaAs buffer layer, a 150-nm-thick spacer layer of $\text{Al}_{0.165}\text{Ga}_{0.835}\text{As}$ was grown with a sheet ($1 \times 10^{12} \text{ cm}^{-2}$) of Si n -type doping (δ -doping) inserted 100 nm from the interface with the GaAs buffer layer. Subsequently, the double-well structure was grown. The layer thickness is given in Fig. 2. On top of the double-well structure

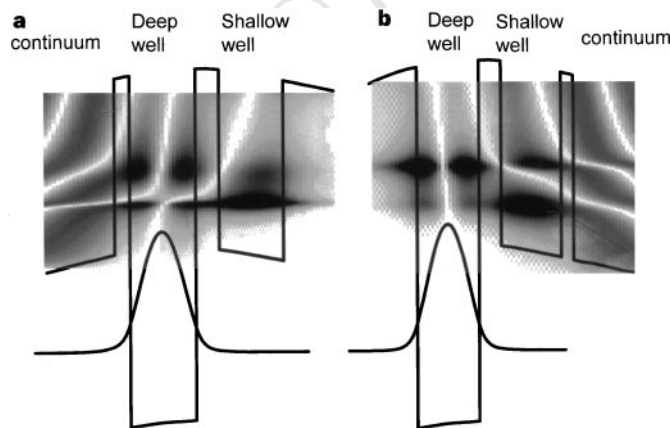


Figure 2 Calculated conduction-band diagram of a portion of the two grown semiconductor structures consisting of a deep 7.3-nm-thick GaAs well coupled to a shallow 6.8-nm-thick $\text{Al}_{0.165}\text{Ga}_{0.835}\text{As}$ well by a 2.5-nm-thick $\text{Al}_{0.33}\text{Ga}_{0.67}\text{As}$ barrier. The two structures differ only in the position of the 1.5-nm-thick $\text{Al}_{0.33}\text{Ga}_{0.67}\text{As}$ tunnel barrier which couples the double quantum well to the continuum. The $\text{Al}_{0.33}\text{Ga}_{0.67}\text{As}$ barrier on the opposite side of the double well is 6.0 nm thick. The growth direction is from right to left. The grey-scale density plot of the calculated squares of the wavefunctions of the two resonances helps in visualizing the effect of the phase shift experienced by the wavefunction in the deep well of structure **a** (sample 9-17-96.1) with increasing energy. This phase shift is responsible for destructive interference in absorption. Its absence in structure **b** (sample 9-17-96.2) leads instead to constructive interference. The modulus squared of the wavefunction of the $n = 1$ states is also displayed (solid curves), calculated self-consistently.

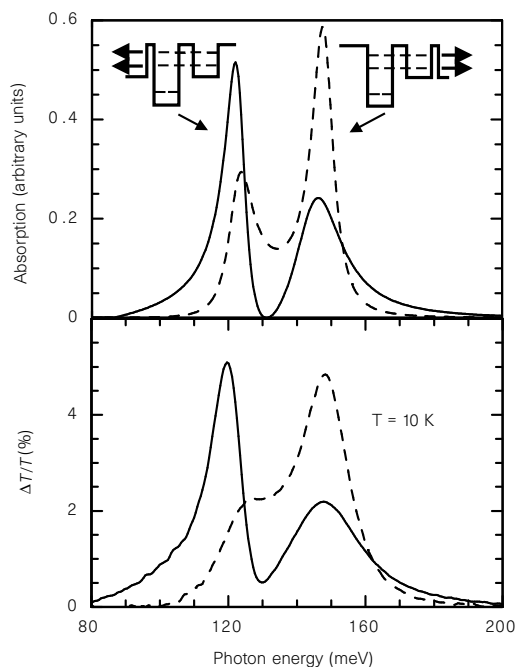


Figure 3 Top: computed absorption for the two structures with the two different locations of the tunnel barrier coupling the step-coupled well to the continuum, as shown by the schematic band diagrams (insets). The location of the tunnel barrier forces destructive (solid line) or constructive (dashed line) interference between the two absorption peaks. Bottom: absorption measurement for the corresponding structures at temperature $T = 10 \text{ K}$. The positions of the absorption peaks correspond to electronic transitions from the ground state to the two resonances. The departure from the calculated lineshapes is attributed to additional broadening mechanisms such as interface roughness scattering which are not included in the model. The dependence of the lineshape on the location of the tunnel barrier is a proof of the interference effect.

(that is, on its left side in Fig. 2a and b), a 231-nm-thick $\text{Al}_{0.165}\text{Ga}_{0.835}\text{As}$ spacer layer was grown with two Si δ -doping layers ($1 \times 10^{12} \text{ cm}^{-2}$), one inserted 22 nm and the other 187 nm from the left edge of the deep well. A 10-nm-thick undoped GaAs region capped the structure. The spacer layer thickness was adjusted to preserve the same distance between the δ -doping layers and the double-quantum-well system, and therefore the electrostatic potentials are identical in both structures. The δ -doping provides a two-dimensional electron gas in the deep well with a calculated sheet electron density of $n_s = 4 \times 10^{11} \text{ cm}^{-2}$.

For the absorption measurements, we processed our samples in a multipass (six) 45° wedge waveguide. This geometry allowed us to couple in linearly polarized radiation with a large component of the polarization normal to the layer (50%) as required by the intersub-band absorption selection rule⁹. The absorption was measured with a Fourier-transform infrared spectrometer (FTIR) using a step-scan modulation technique¹⁰ in which the electron gas in the double well is periodically depopulated by a Ti/Au Schottky barrier contact evaporated on the surface of the sample and the two-dimensional electron gas is contacted by indium balls alloyed into the layer.

The absorption measurements at $T = 10 \text{ K}$ for both structures are compared in Fig. 3 with the results of numerical calculations using the coupled Schrödinger's and Poisson's equations. As predicted, the absorption strength at photon energies between the two resonances is strongly suppressed or enhanced by the interference effect depending on the location of the thin barrier, proving that tunnelling through the latter controls the interference effect when the broadening of the states is dominated by tunnelling. However, the finite broadening introduced by interface disorder prevents full quantum interference; this is the main reason for the departure from the calculated profiles and specifically the reason why the absorption does not vanish in the sample with destructive interference. Indeed, linewidth measurements on samples with the same coupled-well structure but with negligible tunnelling to the continuum showed a full-width at half-maximum of the absorption peaks of $\Gamma = 5 \text{ meV}$. This structure consists of an identical double quantum well between two 60-nm-thick $\text{Al}_{0.33}\text{Ga}_{0.67}\text{As}$ barriers. This value is a measure of the non-tunnelling contribution to the broadening of the optical transitions; it is smaller but not negligible compared with the calculated broadening by tunnelling through the 1.5 nm barrier, $\Gamma_1 \cong \Gamma_2 \cong 16 \text{ meV}$.

Destructive interference in intersub-band absorption in a double-well structure coupled by tunnelling to a continuum has recently been inferred from a fit of the absorption lineshape to a model that included the collision broadening in a phenomenological manner¹¹. The present experiment gives more direct evidence of tunnelling-induced quantum interference by showing that tunnelling can be used to control the sign of the interference.

It is important to stress the difference between the phenomena described here and the Fano interference in intersub-band absorption recently reported by us¹². In that work a minimum in the absorption arises because of interference between matrix elements for the ground state to the continuum and to a single resonance coupled by tunnelling to the same continuum. This leads to a strongly asymmetric absorption lineshape. In contrast, in the phenomena studied here interference arises between absorption paths through two resonances coupled to a continuum, and the direct matrix element from the ground state to the continuum is negligible.

These findings are relevant for the design of semiconductor lasers without population inversion (LWI). Such lasing action has so far been observed only in gases^{4,5}. Essential for LWI is nonreciprocity between emission and absorption. A possible semiconductor LWI scheme would use the quantum-well structure of Fig. 2a for the active regions. The latter would be alternated with electron injectors as in quantum cascade lasers¹³. Electrons would be injected from the thick barrier side at an energy between the two resonances where the

absorption cross-section is a minimum, to ensure strong non-reciprocity between intersub-band absorption and emission^{7,8}. Although the realization of such a laser would be scientifically important, its implementation would be difficult and its technological impact limited by the very short lifetime (a few tenths of picoseconds) of the excited state which is required to achieve strong interference¹⁴. □

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1. Fano, U. Effect of configuration interaction on intensity and phase shifts. *Phys. Rev.* **124**, 1866–1878 (1961).
2. Harris, S. E. Lasers without inversion: interference of lifetime-broadened resonance. *Phys. Rev. Lett.* **62**, 1033–1035 (1989).
3. Arkhipkin, V. G. & Heller, Y. I. Radiation amplification without population at transitions to autoionizing states. *Phys. Lett. A* **98**, 12–14 (1983).
4. Zibrov, S. et al. Experimental demonstration of laser without population inversion via quantum interference in Rb. *Phys. Rev. Lett.* **75**, 1499–1452 (1995).
5. Padmadandu, G. G. et al. Laser oscillation without population inversion in a Sodium atomic beam. *Phys. Rev. Lett.* **76**, 2053–2056 (1996).
6. Boller, K.-J., Imamoglu, A. & Harris, S. E. Observation of electromagnetically induced transparency. *Phys. Rev. Lett.* **66**, 2593–2596 (1991).
7. Imamoglu, A. & Harris, S. E. Lasers without inversion: interference of dressed lifetime-broadened states. *Opt. Lett.* **13**, 1344–1346 (1989).
8. Imamoglu, A. & Ram, R. J. Semiconductor lasers without population inversion. *Opt. Lett.* **19**, 1744–1746 (1994).
9. Levine, B. F., Choi, K. K., Bethea, C. G., Walker, J. & Malik, R. J. New 10 μm infrared detector using intersubband absorption in resonant tunneling GaAlAs superlattices. *Appl. Phys. Lett.* **50**, 1092–1094 (1987).
10. Faist, J. et al. Narrowing of the intersubband electroluminescence spectrum in coupled-quantum-well heterostructures. *Appl. Phys. Lett.* **65**, 94–96 (1994).
11. Smith, H., Campman, K. L., Gossard, A. C. & Imamoglu, A. Tunnel induced transparency: Fano interference in intersubband transitions. *Appl. Phys. Lett.* **70**, 3455–3457 (1997).
12. Faist, J. et al. Tunable Fano interference in intersubband absorption. *Opt. Lett.* **21**, 985–987 (1996).
13. Capasso, F., Faist, J., Sirtori, C. & Cho, A. Y. Infrared (4–11 μm) quantum cascade lasers. *Solid State Commun.* **102**, 231–236 (1997).
14. Kurghin, J. B. & Rosencher, E. Practical aspects of lasing without inversion in various media. *IEEE J. Quant. Electron.* **QE-32**, 1882–1890 (1996).

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Emergence of symbiosis in peptide self-replication through a hypercyclic network

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Symbiosis is an association between different organisms that leads to a reciprocal enhancement of their ability to survive. Similar mutually beneficial relationships can operate at the molecular level in the form of a hypercycle, a collective of two or more self-replicating species interlinked through a cyclic catalytic network^{1–5}. The superposition of cross-catalysis onto autocatalytic replication integrates the members of the hypercycle into a single system that reproduces through a second-order (or higher) form of nonlinear autocatalysis. The hypercycle population as a whole is therefore able to compete more efficiently for existing resources than any one member on its own. In addition, the effects of beneficial mutations of any one member are spread over the entire population. The formation of hypercycles has been suggested as an important step in the transition from inanimate to living chemistry⁶, and a large number of hypercycles are expected to be embedded within the complex networks of living systems⁷. But only one naturally occurring hypercycle has been well documented⁸, while two autocatalytic chemical systems may contain vestiges of hypercyclic organization^{9,10}. Here we report a

chemical system that constitutes a clear example of a minimal hypercyclic network, in which two otherwise competitive self-replicating peptides symbiotically catalyse each others' production.

The present design of a minimal hypercycle is based on two self-replicating coiled coil peptides **R**₁ and **R**₂ (Fig. 1). The replicator **R**₁ was recently reported^{11,12} and is produced as the ligation product of the electrophilic peptide fragment **E** and the nucleophilic fragment **N**₁. The replicator **R**₂ is made from the same electrophilic fragment but a different nucleophilic peptide fragment **N**₂. The nucleophilic fragments **N**₁ and **N**₂ differ in their sequence at the hydrophobic recognition surface—**N**₁ is composed of valine and leucine whereas **N**₂ is made up of isoleucine and leucine residues. This difference in sequence at the hydrophobic core is known to affect profoundly the aggregation state of coiled coils^{13,14}. Furthermore it is known that conservative mutations in this region of the structure can drastically alter the kinetic behaviour of the replicator^{11,12,15}.

The ability of **R**₂ to self-replicate was determined by observation of characteristics previously established as signatures of self-replication (Fig. 2)^{11,12}. Similar to that of **R**₁, the new replicator **R**₂ also displays a parabolic growth profile. Numerical fitting of the kinetic data obtained for **R**₂ to the empirical rate equations of von Kiedrowski¹⁶ gave a background rate constant $k_b = 0.072 \pm 0.005 \text{ M}^{-1} \text{ s}^{-1}$ and an apparent autocatalytic rate constant $k_a = 52 \pm 1 \text{ M}^{-3/2} \text{ s}^{-1}$, making **R**₂ more efficient than its relative **R**₁ ($k_b = 0.063 \text{ M}^{-1} \text{ s}^{-1}$ and constant $k_a = 29.4 \text{ M}^{-3/2} \text{ s}^{-1}$).

A solution containing all three fragments **E**, **N**₁ and **N**₂ gave a combinatorial synthesis of both replicators. *A priori*, one would

expect a survival-of-the-fittest situation where the more efficient replicator **R**₂ would overwhelm **R**₁ by consuming the common fragment **E** more quickly. At first glance, this expectation seemed to be borne out as **R**₂ was produced in greater abundance than **R**₁ (as expected, when molecular interactions are disrupted in the presence of guanidinium hydrochloride, no kinetic preference for **R**₂ over **R**₁ was observed). However, the situation is more interesting and complex. When we sought to give **R**₁ an advantage in this competition by adding 40% **R**₁ (with respect to the nucleophile concentration) at the start of the reaction, to our surprise the rate of **R**₁ self-production increased by only 1.7 times over the unseeded reaction but the rate of **R**₂ formation was enhanced to a greater extent, by 5.4 times (Table 1, Fig. 3). Thus the two replicators are not mutually exclusive in their growth; **R**₁ catalyses the formation of **R**₂ as well as itself. Likewise, perturbation of the reaction by seeding it with 45% **R**₂ not only increased the rate of **R**₂ production 2.9 times but **R**₁ as well, by 3.5 times. Thus a cross-catalytic cycle is cooperatively coupled with two self-replicating reactions, making this system one which is hypercyclic in nature. There are four characteristic outcomes expected for such a hypercyclic network, depending on the relative efficiencies of the coupled catalytic and autocatalytic reactions². The observed greater efficiencies of the catalytic reactions over the autocatalytic components of the system are the most desirable outcomes which assure the stability of the hypercycle: production of one species promotes the production of the other to an even greater degree. This particular mode of catalytic coupling prevents one replicator from overwhelming the other and enables the two to reproduce as a single coherent unit.

To verify that **R**₁ and **R**₂ catalyse each other's production, the

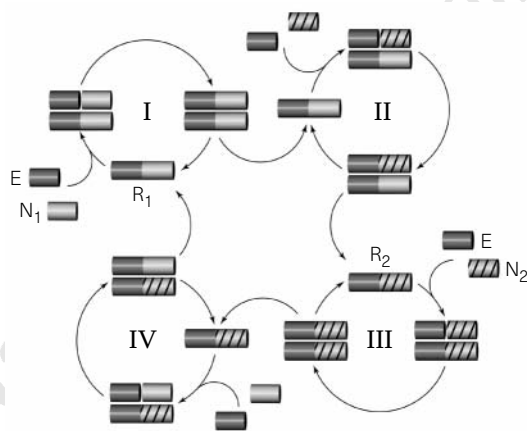


Figure 1 Schematic diagram of a minimal hypercycle based on two self-replicating peptides. Cycles I and III show the self-producing cycles of replicators **R**₁ (dark grey/light grey) and **R**₂ (dark grey/striped) respectively, which pre-organize their constituent fragments thereby promoting peptide ligation. Cycle II, where **R**₁ promotes **R**₂ formation, and cycle IV, where **R**₂ promotes **R**₁ formation, comprise the catalytic components of the hypercycle and allow the replicators to positively regulate each others' production. The mechanistic details of the present hypercyclic network may be more complex than the minimal system depicted here. Detailed kinetic analyses of the replicator sequences have shown that the autocatalytically productive intermediates involve, at least in part, quaternary complexes in which two template strands pre-organize the reactive peptide fragments (ref. 12 and K. Kumar, D.H.L., M.R.G., unpublished results). The following peptide sequences were employed in this study: replicator 1 (**R**₁), ArCONH-RMKQLEEKVYELLKSKVA-CLEXEVARLKKLVGE-CONH₂; replicator 2 (**R**₂), ArCONH-RMKQLEEKVYELLKSKVA-CLEXEIARLKKLIGE-CONH₂; electrophilic fragment (**E**), ArCONH-RMKQLEEKVYELLKSKVA-COSBn; nucleophilic fragment 1 (**N**₁), H₂N-CLEXEVARLKKLVGE-CONH₂; nucleophilic fragment 2 (**N**₂), H₂N-CLEXEIARLKKLIGE-CONH₂. Bn, benzyl; Ar, 4-acetamidophenyl; and X, lysine-ε-NHCO-Ar.

Table 1 Initial rates of product formation

Product	No replicators added	+40% R ₁	+45% R ₂
R ₁	4.8	8.2	17.0
R ₂	5.8	31.1	16.9

The data in this table (in units of $10^{-6} \text{ M min}^{-1}$) are for reactions containing the three peptide fragments in the absence and presence of added replicators.

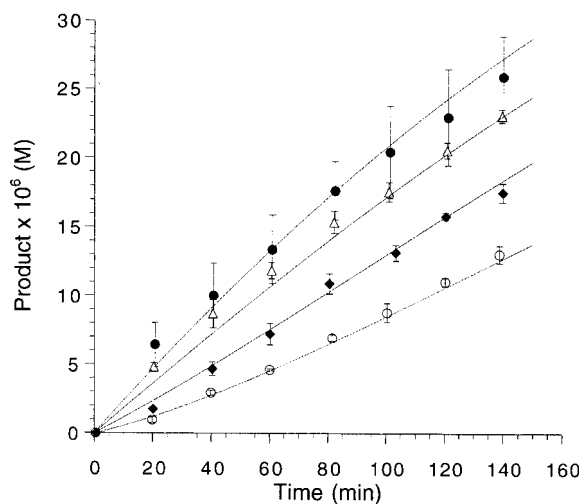


Figure 2 Production of **R**₂ as a function of time in the presence of various initial concentrations of **R**₂. Open circles, in the absence of any added **R**₂; filled diamonds, in the presence of 4.0 μM; open triangles, in the presence of 21.4 μM; and filled circles, 42.6 μM of initially added **R**₂. Curves were generated by nonlinear least-squares fit of the data to the empirical rate equation of von Kiedrowski using the program SimFit¹⁶. Data are an average of two experiments.

reaction mixtures were simplified to include **E** and only one nucleophile, and then seeded with the template that was not produced *in situ* (Fig. 4). Comparisons with unseeded reactions revealed that even in these simplified systems one template can promote the formation of the other, giving rate enhancements much larger than what would be expected if the reaction mixture were seeded with the autocatalytic template. Reaction mixtures containing **E** and **N**₁ that were seeded with 25% **R**₁ enhanced the initial rate of production of **R**₁ from $3.9 \times 10^{-8} \text{ M min}^{-1}$ to $1.5 \times 10^{-7} \text{ M min}^{-1}$, a 3.8 times increase over the unseeded reaction. Seeding of the same reaction mixture with 25% **R**₁ would improve the rate by only 2.8 times. Similarly, seeding reaction mixtures containing **E** and **N**₂ with 35% **R**₁ gave a 5.4 times rate enhancement over the $5.0 \times 10^{-8} \text{ M min}^{-1}$ rate observed for the reaction without added catalyst. The increase is greater than the 3.6

times enhancement expected for the autocatalytic reaction containing 35% **R**₂.

We now consider the sequence selectivity issues in the formation of the hypercyclic peptide network. The operation of the hypercycle is based on complementary, as well as self-complementary, forms of catalysis. As noted below, there is mounting evidence that both processes are strongly sequence selective. Previously we had shown that in the case of replicator **R**₁, even conservative mutations (Val9Ala—where a valine has been substituted by an alanine at position 9—and Leu26Ala) in the hydrophobic core residues completely abolish the autocatalytic process^{11–12}. In this study we have determined that similar replicator **R**₂ mutations are also autocatalytically infertile. There is also good evidence for high sequence selectivity in the cross-catalytic component of the system. Control studies have indicated that the Leu26Ala **R**₂ mutant cannot cross-catalyse the formation of replicators **R**₁ nor **R**₂. Although in a recent study¹⁵ we have shown that the Val9Ala **R**₁ mutant can efficiently cross-catalyse the formation of **R**₁, we have found it to be ineffective in catalysing **R**₂ production. Moreover, in a related study we have shown that diminution in the initial rate of peptide fragment condensation of more than 3 orders of magnitude can be caused even by electrostatic substitutions at the solvent-exposed *e* and *g* positions of the heptad repeat sequence¹⁷. Although the above studies strongly support high sequence selectivity in the catalytic and autocatalytic components of the hypercyclic network, a significantly large sequence-space must undoubtedly exist that would enable the spontaneous self-organization of even more complex networks. Studies along those lines are under investigation.

The work reported here may have particular relevance to various origin-of-life theories^{1–4,18}. It has been suggested that at the dawn of life the onset of darwinian evolution must have been marked by

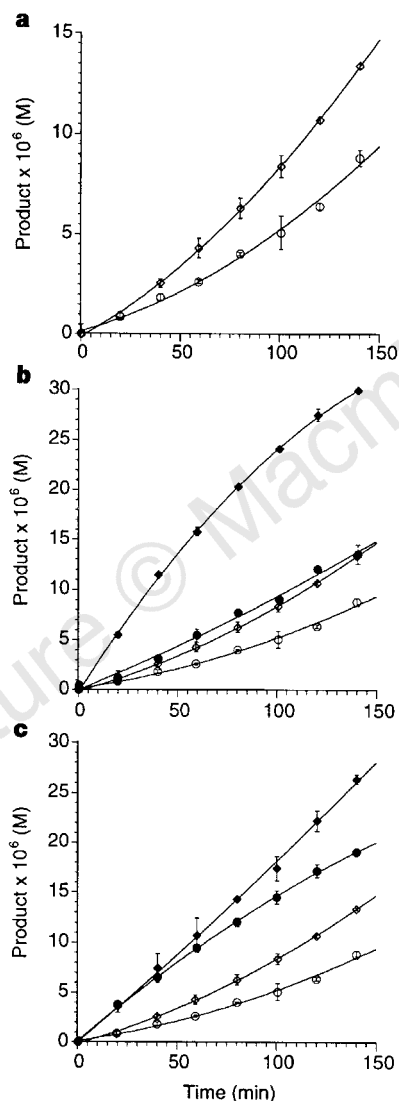


Figure 3 Replicators **R**₁ and **R**₂ self-organize into a two-membered hypercyclic network. **a**, Production of **R**₁ (empty circles) and **R**₂ (empty diamonds) as a function of time for reaction mixtures containing **E**, **N**₁ and **N**₂. **b**, Formation of **R**₁ (filled circles) and **R**₂ (filled diamonds) as a function of time for reaction mixtures containing the three fragments and 40% **R**₁. **c**, Formation of **R**₁ (filled circles) and **R**₂ (filled diamonds) as a function of time for reaction mixtures containing the three fragments and 45% **R**₂. In **b** and **c**, production formation in the absence of added templates are shown for comparison. Data are an average of two experiments. Curves are shown to guide the eye.

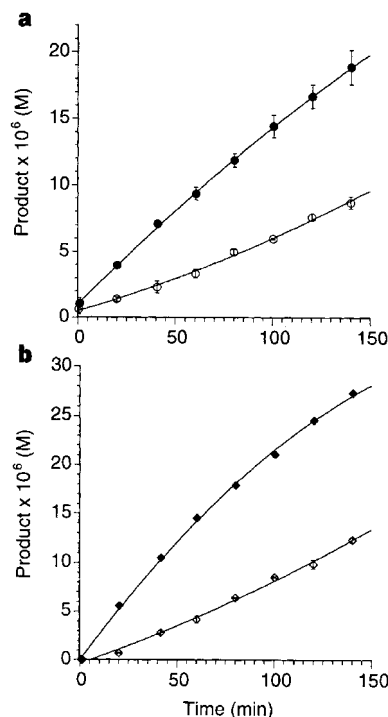


Figure 4 Replicators **R**₁ and **R**₂ are cross-catalytic. **a**, Formation of **R**₁ as a function of time for the reaction mixture containing only **E** and **N**₁ in the absence (empty circles) and in the presence (filled circles) of 35% **R**₂. **b**, Formation of **R**₂ as a function of time for the reaction mixture containing **E** and **N**₂ in the absence (empty diamonds) and in the presence (filled diamonds) of 25% **R**₁. Data are an average of two experiments. Curves are shown to guide the eye.

selection based on feedback processes of genotype replication¹⁹. It is also likely that molecular genotypes and phenotypes may have been the very same molecules²⁰. Our example of a hypercyclic peptide network supports the idea that peptides could play a role in both hypotheses. □

Methods

Self-replication of R₂. All reactions were done in 0.6 ml Eppendorf tubes at 23 °C. A stock solution containing E, N₂ and the internal standard 4-acetamidobenzoic acid (ABA), were seeded with various amounts of R₂. Benzylmercaptan (1 µl) was then added. Reactions were initiated by adding 3-(N-morpholino)propanesulphonic acid (MOPS) buffer (pH = 7.50, 200 mM, 236 µl), giving a total volume of 300 µl and concentrations of [N₂] = 104.5 µM, [E] = 94.2 µM, [R₂] = 0, 4.0, 21.4 or 42.6 µM. [MOPS] = 157 mM, [ABA] = 40.4 µM. Samples (30 µl) were taken at various time points and quenched with 2% trifluoroacetic acid (TFA) in water (70 µl) then stored at -70 °C. Samples were analysed by high pressure liquid chromatography on a Zorbax C8 column using an acetonitrile/water/0.1% TFA gradient while monitoring at 270 nm. The identity of all peptides was determined by mass spectrometry and verified by coinjection with authentic samples. Experiments were done in duplicate.

Determination of hypercyclic organization in the E/N₁/N₂ mixture. Reactions were done as described above except that the stock solution contained, besides E and ABA, both N₁ and N₂, which was subsequently seeded with either R₁, R₂, or water. Reactions were initiated by adding MOPS buffer (pH = 7.50, 200 mM, 236.6 µl), giving a total volume of 300 µl and concentrations of [N₁] = 112.5 µM, [N₂] = 112.7 µM, [E] = 91.1 µM, [MOPS] = 157.7 mM, [ABA] = 97.1 µM, [R₁] = 45.1 µM, [R₂] = 50.4 µM.

Verification of the catalytic components of the hypercycle. Reactions were performed as described above except only one nucleophile was present in the reaction mixture and the reaction was seeded with the replicator that was not produced *in situ*. Initial concentrations are (1) [E] = 88.9 µM, [N₁] = 98.2 µM, [R₂] = 25.2 µM, [ABA] = 50.5 µM; (2) [E] = 80.4 µM, [N₂₁] = 96.9 µM, [R₁] = 35.3 µM, [ABA] = 36.9 µM.

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1. Eigen, M. & Schuster, P. The hypercycle. A principle of natural self-organization. Part A: emergence of the hypercycle. *Naturwissenschaften* **64**, 541–565 (1977).
2. Eigen, M. & Schuster, P. The hypercycle. A principle of natural self-organization. Part B: the abstract hypercycle. *Naturwissenschaften* **65**, 7–41 (1978).
3. Eigen, M. & Schuster, P. The hypercycle. A principle of natural self-organization. Part C: The realistic hypercycle. *Naturwissenschaften* **65**, 341–369 (1978).
4. Eigen, M. Self-organization of matter and the evolution of biological macromolecules. *Naturwissenschaften* **58**, 465–523 (1971).
5. Müller-Herold, U. What is a hypercycle? *J. Theor. Biol.* **102**, 569–584 (1983).
6. Lee, D. H., Severin, K. & Ghadiri, M. R. Autocatalytic networks: the transition from molecular self-replication to molecular ecosystems. *Curr. Opin. Chem. Biol.* (in the press).
7. Ricard, J. & Noat, G. Electrostatic effects and the dynamics of enzyme reactions at the surface of plant cells. I. A theory of the ionic control of a complex multi-enzyme system. *Eur. J. Biochem.* **155**, 183–190 (1986).
8. Eigen, M., Biebricher, C. K., Gebinoga, M. & Gardiner, W. C. The hypercycle. Coupling of RNA and protein biosynthesis in the infection cycle of an RNA bacteriophage. *Biochemistry* **30**, 11005–11018 (1991).
9. Hong, J.-I., Feng, Q., Rotello, V. & Rebek, J. Jr Competition, cooperation and mutation: improving a synthetic replicator by light irradiation. *Science* **255**, 848–850 (1992).
10. Achilles, T. & von Kiedrowski, G. A self-replicating system from three starting materials. *Angew. Chem. Int. Edn Engl.* **32**, 1198–1201 (1993).
11. Lee, D. H., Granja, J. R., Martinez, J. A., Severin, K. S. & Ghadiri, M. R. A self-replicating peptide. *Nature* **382**, 525–528 (1996).
12. Severin, K. S., Lee, D. H., Martinez, J. A. & Ghadiri, M. R. Peptide self-replication via template-directed ligation. *Chem. Eur. J.* **3**, 1017–1024 (1997).
13. Harbury, P. B., Zhang, T., Kim, P. S. & Alber, T. A switch between two-, three- and four-stranded coiled coils in GCN4 leucine zipper mutants. *Science* **262**, 1401–1407 (1993).
14. Hu, J. C., O'Shea, E. K., Kim, P. S. & Sauer, R. T. Sequence requirements for coiled coils: analysis with λ repressor-GCN4 leucine zipper fusions. *Science* **250**, 1400–1403 (1990).
15. Severin, K., Lee, D. H., Martinez, J. A. & Ghadiri, M. R. Dynamic error-correction in an autocatalytic peptide network. *Angew. Chem. Int. Edn Engl.* (in the press).
16. von Kiedrowski, G. Minimal replicator theory I: parabolic versus exponential growth. *Bioorg. Chem. Front.* **3**, 113–146 (1993).
17. Severin, K., Lee, D. H., Kennan, A. J. & Ghadiri, M. R. A synthetic peptide ligase. *Nature* **389**, 706–709 (1997).
18. Kauffman, S. A. *The Origins of Order* (Oxford Univ. Press, New York, 1993).
19. Küppers, B.-O. *The Origin of Biological Information* (MIT Press, Cambridge, MA, 1990).
20. Joyce, G. F. RNA evolution and the origins of life. *Nature* **338**, 217–224 (1989).

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Kinetic limitations on droplet formation in clouds

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The 'indirect' radiative cooling of climate due to the role of anthropogenic aerosols in cloud droplet formation processes (which affect cloud albedo) is potentially large, up to -1.5 W m^{-2} (ref. 1). It is important to be able to determine the number concentration of cloud droplets to within a few per cent, as radiative forcing as a result of clouds is very sensitive to changes in this quantity², but empirical approaches are problematic^{3–5}. The initial growth of a subset of particles known as cloud condensation nuclei and their subsequent 'activation' to form droplets are generally calculated with the assumption that cloud droplet activation occurs as an equilibrium process described by classical Köhler theory^{6,7}. Here we show that this assumption can be invalid under certain realistic conditions. We conclude that the poor empirical correlation between cloud droplet and cloud condensation nuclei concentrations is partly a result of kinetically limited growth before droplet activation occurs. Ignoring these considerations in calculations of total cloud radiative forcing based on cloud condensation nuclei concentrations could lead to errors that are of the same order of magnitude as the total anthropogenic greenhouse-gas radiative forcing¹.

Cloud droplet activation and subsequent treatments of cloud droplet growth in atmospheric models generally rely on the assumption that pre-activation growth is accurately described by an equilibrium model in which the particle diameter is always at equilibrium with the local supersaturation^{6,7}. The equilibrium relationship between supersaturation and particle size for a particle composed of highly soluble inorganic species can be described by the well-known Köhler equation (curve A, Fig. 1)⁸. Cloud droplet nuclei (CDN) activate when they grow larger than their critical diameter, D_{pc} , after which they can grow spontaneously, limited only by growth kinetics. The concept of CDN is distinct from that of CCN in that, whereas CCN are defined as those particles that activate to become cloud droplets within a cloud chamber of fixed or prescribed supersaturation, CDN are those particles that actually activate in the atmosphere under conditions of time-varying supersaturation.

To evaluate the conditions under which the equilibrium activation model is valid, two timescales will be defined. One is the timescale for particle growth that would be required for that particle to remain at equilibrium as the ambient supersaturation ratio increases in a rising air parcel, τ_e . The other is the timescale for actual change in the droplet size resulting from condensational growth, τ_g . Hence, if $\tau_e \gg \tau_g$ then the equilibrium model is reasonable; otherwise, CDN activation, and hence the cloud droplet size distribution, can be accurately predicted only if the kinetics of droplet growth are considered. To calculate τ_e , the rate of change of the droplet diameter that would be required for that droplet to remain at its equilibrium size, dD_{pe}/dt , is determined from the combination of two effects. First, the time rate of change of supersaturation, dS/dt , can be determined using a simple one-dimensional adiabatic parcel model⁹. Next, the rate of change of D_{pe} with respect to supersaturation, dD_{pe}/dS , is determined by differentiating

the Köhler relationship. Combining these two expressions yields $dD_{pe}/dt = (dD_{pe}/dS)(dS/dt)$, after which τ_e can be determined from $\tau_e = D_{pe}/(dD_{pe}/dt)$. Similarly, the time rate of change of the particle diameter resulting from condensational growth, dD_{pg}/dt , can be computed using established expressions^{10,11}, after which τ_g can be determined from $\tau_g = D_{pg}/(dD_{pg}/dt)$.

Figure 2 shows the two timescales as a function of particle critical supersaturation S_c . The base-case parameters given in the figure were estimated for typical marine stratiform clouds⁹, which are climatically the most important cloud type². The most notable feature of Fig. 2 is that for $S_c < 0.042\%$, $\tau_g \gg \tau_e$; that is, these pre-activated droplets do not grow sufficiently quickly to follow the changes in the equilibrium diameter, implying that for particles with $S_c < 0.042\%$ (for example, pure NH_4HSO_4 particles of size $>0.23 \mu\text{m}$ dry diameter), condensational growth kinetics are important. The fraction of CCN in this regime can be estimated using the common empirical parameterization of the cumulative CCN spectrum, $N = CS^k$, where N is the CCN number concentration, S is the supersaturation, and C and k are empirically determined parameters¹². If the peak supersaturation achieved in an air parcel is S_{max} , the fraction of CCN for which growth kinetics are important is $(S_c^*/S_{\text{max}})^k$, where S_c^* is the value of S_c at which $\tau_g = \tau_e$. When this fraction is significant, accurately predicting cloud droplet size distributions requires a kinetic description of CDN pre-activation growth. Assuming a value of S_{max} of 0.1%, this fraction is 0.76, 0.63 and 0.53 for k values of 0.3, 0.5, and 0.7, respectively, for the base case of Fig. 2. These exponents are representative of data from observations of marine stratiform clouds¹².

The sensitivity of S_c^* to temperature, pressure, droplet diameter, updraft velocity, and thermal and mass accommodation coefficients (these coefficients represent the fractions of gas-particle collisions that result in actual transfer) is presented in Fig. 3. The base case is seen actually to be a conservative estimate of S_c^* , as almost all the values in Fig. 3 lie above $S_c^* = 0.042\%$. Changes in pressure and temperature do not greatly affect S_c^* . Changes in the updraft velocity are more significant, as dS/dt is directly proportional to this quantity. S_c^* is sensitive to changes in the accommodation coefficients only if α_c or α_t is less than 0.1. Values for α_c in the range of 0.03 to 1.0 have been reported¹³. S_c^* is most sensitive to the particle diameter (increasing by more than a factor of 2 for a 50% change in diameter)

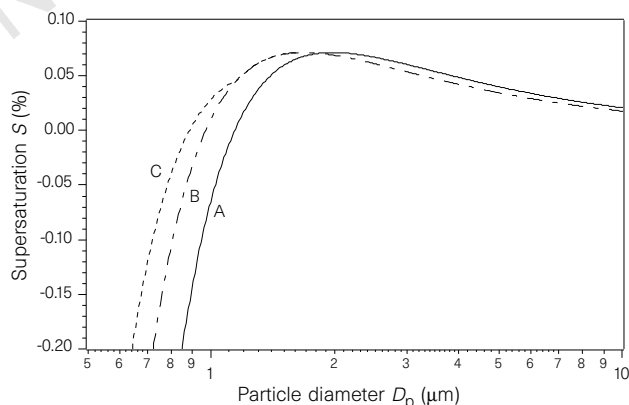


Figure 1 Köhler curves for three different particles. All curves are for $S_c = 0.071\%$. For curve A, $\sigma = \sigma_{\text{water}}$, and the solute is infinitely soluble. For curve B, σ is lower because of the presence of an organic species, so $\sigma = 0.85 \sigma_{\text{water}}$, but the solute is still infinitely soluble. Curve C is as curve B, except the organic solubility is assumed to be 0.02M. D_{pc} shifts from 1.97 μm in curve A to 1.67 μm in curves B and C because of the surface tension decrease. Curves B and C join together when the organic fraction of curve C fully dissolves.

primarily because τ_g is directly proportional to D_p^2 . This implies that a particle that began in the equilibrium regime may, through condensational growth, cross over into the kinetic regime while it is still unactivated, indicating that the fraction of particles for which growth kinetics is important is even higher than estimated in the base case.

There is initial evidence that organic compounds make up a significant fraction of CCN mass^{14,15} and can alter a particle's Köhler curve¹⁶. The effect of organics on τ_e and τ_g can be divided into three distinct factors: (1) changes in the droplet surface tension; (2) gradual dissolution of solute due to limited solubility; and (3) changes in the mass accommodation coefficient.

The presence of water-soluble organics decreases in varying amounts the surface tension of these solutions¹⁶. In the absence of any other perturbations, a decrease in the droplet surface tension σ by $\Delta\sigma/\sigma$ can be shown to decrease the critical diameter by a factor of $\Delta\sigma/\sigma$, as illustrated by curves A and B in Fig. 1. One consequence of this decrease is to increase τ_e because at a given value of S , curve B is shifted to smaller sizes relative to curve A, which causes a decrease in dD_{pe}/dS . Another consequence is that τ_g decreases by roughly $2\Delta\sigma/\sigma$ because τ_g is directly proportional to D_p^2 . The combination of these effects shifts S_c^* to smaller values. Using the base-case parameters, for $\Delta\sigma/\sigma = 15\%$, τ_e increases by about 15%, τ_g decreases by 28%, and S_c^* decreases by 11%. As a result, the fraction of particles in the kinetic regime decreases by 6%.

If the solubility limit of the organic fraction in a droplet is reached, then the amount of solute within that droplet increases as the droplet grows in order to maintain saturated conditions. Qualitatively, the gradual dissolution of organics broadens the Köhler curve as illustrated in curve C, Fig. 1. In some cases, local maxima and minima can arise in the Köhler curve when organic components fully dissolve¹⁶. The overall effect of gradual dissolution on both time constants seems to be small compared with surface tension effects for realistic conditions. We do not consider here a possibly related influence, the kinetics of dissolution.

It has already been shown that S_c^* changes rapidly with changes in the condensation coefficient α_c for $\alpha_c \leq 0.1$ as a result of changes in τ_g . Many organic compounds can be present at the solution/air interface in quantities in excess of the bulk average as a result of their partially hydrophobic nature¹⁷. Organics can change the evaporative

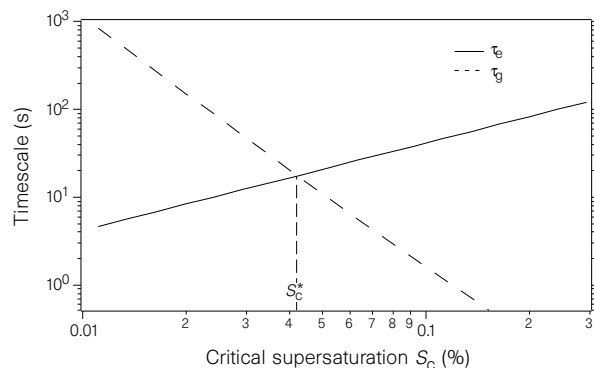


Figure 2 Base-case comparison of the equilibrium (τ_e) and droplet growth (τ_g) timescales as a function of critical supersaturation. Base-case parameters are temperature $T = 280 \text{ K}$, pressure $p = 900 \text{ mbar}$, updraft velocity $u = 20 \text{ cm s}^{-1}$, and mass (α_c) and thermal (α_t) accommodation coefficients equal to unity. To calculate the timescales, a diameter must be specified; the equilibrium diameter at 100% relative humidity was chosen for the base case. It can be seen that S_c^* is a good indicator of the transition from kinetic to equilibrium regimes because the ratio τ_e/τ_g is a strong function of critical supersaturation. Note that there is an inverse relationship between S_c and particle diameter. For example, an ammonium bisulphate particle with $S_c = 0.01\%$ has a dry diameter of 0.6 μm , and for $S_c = 0.2\%$, dry particle diameter is 0.09 μm .

properties of bulk water even in minute quantities¹⁸. Therefore, it seems plausible at least that organics could cause substantial changes in α_c , and therefore S_c^* , although no attempt at quantifying such an effect will be made here.

There do not seem to be any observations of CDN and cloud droplets where the full set of possible controlling variables has been measured. It may not in fact be possible at present to make the necessary measurements *in situ* that could support or negate the above model calculations. However, there is evidence that cloud droplet growth can be delayed in cloud chambers¹⁹. The effect has been attributed to the presence of an organic coating¹⁹, but kinetic variations of activation could also explain these observations.

Neglecting kinetic limitations on pre-activation droplet growth has direct consequences for models of cloud droplet populations, and therefore cloud radiative climate forcing. First, models that use an equilibrium assumption such as $N = CS^k$ must overestimate the droplet number concentration that actually would form, because kinetically limited growth means that some of those droplets fail to activate. To estimate the error in cloud droplet concentration and hence the magnitude by which the equilibrium activation model overestimates cloud radiative forcing, we compare results from the approximation $N = CS^k$ with those from a one-dimensional adiabatic cloud model that includes explicit pre-activation growth kinetics⁹. Using the above base case, and for $k = 0.3, 0.5$ and 0.7 , the equilibrium model estimates CDN concentrations ($[CDN]$) to be 69%, 29% and 14%, higher respectively, than the cloud model. These differences in $[CDN]$ can be translated into estimates of the overprediction of globally averaged cloud forcing using a previously published relationship between global-mean cloud radiative forcing and $[CDN]$ changes². On the basis of this calculation, the equilibrium activation model overestimates the magnitude of the total (natural plus anthropogenic) cloud radiative forcing by 3.6, 1.8 and 0.9 W m^{-2} , respectively, which are significant compared with the total estimated greenhouse forcing¹ of 2.4 W m^{-2} . Although these numbers are approximate, it is clear from their magnitude that climate models must consider activation kinetics in order to accurately predict the radiative climate forcing by clouds.

Another consequence of kinetically limited activation is that CCN chambers that have residence times that differ from typical growth times found in real clouds will incorrectly measure the CDN concentration. Figure 2 shows typical activation timescales between a fraction of a second and hundreds of seconds, whereas cloud chambers tend to have residence times of a few seconds to a few tens

of seconds. The measured CCN concentration will be either too small or too large, depending on whether the CCN instrument had a growth time that was shorter or longer than that in the actual cloud. In either case, the data will not be appropriate for use with climate models unless the discrepancy is considered. CCN measurements under conditions that mimic cloud conditions would yield data more useful for calculations of cloud radiative forcing. □

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- Intergovernmental Panel on Climate Change *Climate Change 1995: The Science of Climate Change* (eds Houghton, J. T. et al.) 3–7 (Cambridge Univ. Press, Cambridge, 1996).
- Charlson, R. J. et al. Climate forcing by anthropogenic aerosols. *Science* **255**, 423–430 (1992).
- Twomey, S. & Warner, J. Comparison of measurements of cloud droplets and cloud nuclei. *J. Atmos. Sci.* **24**, 702–703 (1967).
- Hegg, D. A., Ferek, R. J. & Hobbs, P. V. Light-scattering and cloud condensation nucleus activity of sulfate aerosol measured over the Northeast Atlantic Ocean. *J. Geophys. Res.* **98**, 14887–14894 (1993).
- Pueschel, R. F. et al. Aerosols in polluted versus nonpolluted air masses—long-range transport and effects on clouds. *J. Clim. Appl. Meteorol.* **25**, 1908–1917 (1986).
- Schwartz, S. E. et al. in *Aerosol Forcing of Climate* (eds Charlson, R. J. & Heintzenberg, J.) 251–280 (Wiley, Chichester, 1995).
- Ghan, S. J. et al. A parameterization of cloud droplet nucleation part I: single aerosol type. *Atmos. Res.* **30**, 197–221 (1993).
- Seinfeld, J. H. *Atmospheric Chemistry and Physics of Air Pollution* (Wiley, New York, 1986).
- Jensen, J. B. & Charlson, R. J. On the efficiency of nucleation scavenging. *Tellus B* **36**, 367–375 (1984).
- Pruppacher, H. R. & Klett, J. D. *Microphysics of Clouds and Precipitation* (Reidel, Dordrecht, 1978).
- Fukuta, N. & Walter, A. Kinetics of hydrometeor growth from a vapor-spherical model. *J. Atmos. Sci.* **27**, 1160–1172 (1970).
- Hobbs, P. V. in *Aerosol–Cloud–Climate Interactions* (ed. Hobbs, P. V.) 33–37 (Academic, San Diego, 1993).
- Mozurkewich, M. Aerosol growth and the condensation coefficient for water: a review. *Aerosol Sci. Technol.* **5**, 223–236 (1986).
- Novakov, T. & Penner, J. E. Large contribution of organic aerosols to cloud-condensation-nuclei concentrations. *Nature* **365**, 823–826 (1993).
- Rivera-Carpio, C. A. et al. Derivation of contributions of sulfate and carbonaceous aerosols to cloud condensation nuclei from mass size distributions. *J. Geophys. Res.* **101**, 19483–19493 (1996).
- Shulman, M. L. et al. Dissolution behavior and surface tension effects of organic compounds in nucleating cloud droplets. *Geophys. Res. Lett.* **23**, 227–280 (1996).
- Stumm, W. & Morgan, J. J. *Aquatic Chemistry* (Wiley, New York, 1981).
- Barnes, G. T. & La Mer, V. K. in *Retardation of Evaporation by Monolayers: Transport Processes* (ed. La Mer, V. K.) 9–33 (Academic, New York, 1962).
- Bigg, E. K. Discrepancy between observation and prediction of concentrations of cloud condensation nuclei. *Atmos. Res.* **20**, 82–86 (1986).

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Vanishing atomic migration barrier in SiO_2

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Understanding the high-pressure behaviour of SiO_2 , a prototypical network-forming material, is important for resolving many problems in the Earth sciences. For pressures of 1–3 GPa ($\sim 1\text{--}3 \times 10^4$ atm), it has been shown that increases in pressure result in higher rate constants for atomic transport processes such as diffusion, viscous flow and crystal growth in SiO_2 as well as in some silicate melts^{1–5}. Structural transitions and coordination changes observed beyond 10 GPa (refs 5–9) may also be related to this pressure-induced increase in atomic mobility. There must be limits, however, on the extent to which pressure can enhance mobility, as a migration barrier decreasing linearly with pressure should vanish at a critical pressure, beyond which a sudden change in behaviour should be observed^{10,11}. Here we report measurements of the pressure dependence of the growth rate of quartz from amorphous SiO_2 for pressures up to 6 GPa. We observe a sharp peak in growth rate—implying a minimum in viscosity—at 3 GPa, which we interpret as evidence that the critical pressure is being traversed. The corresponding depth below the Earth's surface at which this peak occurs (~ 100 km)

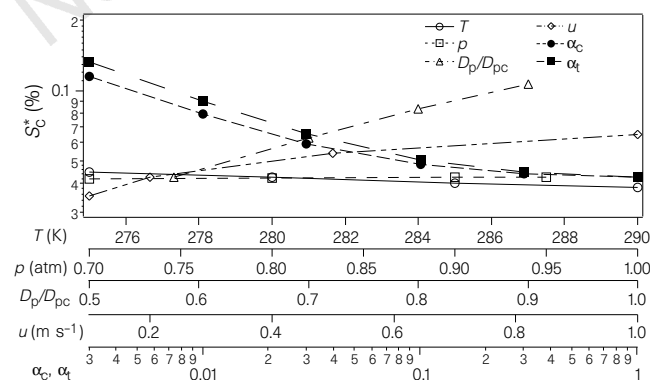


Figure 3 A study of the sensitivity of S_c^* to various parameters. The base case value is 0.042%, which is lower than most of the values plotted. Temperature and pressure cause the smallest changes, whereas updraft velocity and the accommodation coefficients are more important. The specified diameter causes the greatest change in the value of S_c^* . D_{pc} is the critical diameter; that is, the size at which the particle is considered to be activated. The base case, where D_p is that at 100% relative humidity, corresponds to $D_p/D_{pc} = 0.577$.

suggests that this critical pressure may be related to the ubiquitous cut-off in subduction-related volcanism observed when oceanic plates reach roughly this depth.

The relationship between crystal growth rate ν and viscosity η or diffusivity D of network formers has been well established¹², especially for oxides¹³:

$$\nu = f(D/\lambda)[1 - \exp(n\Delta G/kT)] \quad (1)$$

where f is the fraction of interfacial sites at which the growth reaction can occur, D is the diffusivity, λ is the bond length, ΔG is the change in Gibbs free energy per molecular unit crystallized, and n is the number of units crystallized per thermally produced defect at the interface before defect annihilation. D can be replaced by the inverse of the viscosity, η , through the Stokes–Einstein relation $\eta = kT/3\pi\lambda D$. Thus a measurement of ν gives us insight into the behaviour of other atomic transport processes such as diffusion and flow.

Crystallization experiments were done in a multi-anvil solid-medium apparatus¹⁴ at 1,673 K and 2.5–8.0 GPa pressure (P). The crystal growth morphology is similar to that found by Fratello *et al.*². Measured growth rates at 2.5 GPa coincide with those measured by Fratello *et al.* for this material and the rates obtained at 3 GPa are consistent with an extrapolation of their data obtained between 0.5 and 2.5 GPa (Fig. 1), following the exponential increase of ν with P . In marked contrast, ν decreases with P from 3 to 6 GPa. The pressure dependence of the rate is characterized by the apparent activation volume, $\Delta V^* = -kT(\partial \ln \nu / \partial P)_T$ (solid lines in Fig. 1). The data for $P \leq 3$ GPa are fitted by $\Delta V^* = -19.2 \pm 1.5 \text{ cm}^3 \text{ mol}^{-1}$; the data for 3–6 GPa are fitted by $\Delta V^* = +5.2 \pm 1.6 \text{ cm}^3 \text{ mol}^{-1}$.

The change in behaviour at 3 GPa might be due to either of the bracketed factors in equation (1). Fratello *et al.*² assumed that

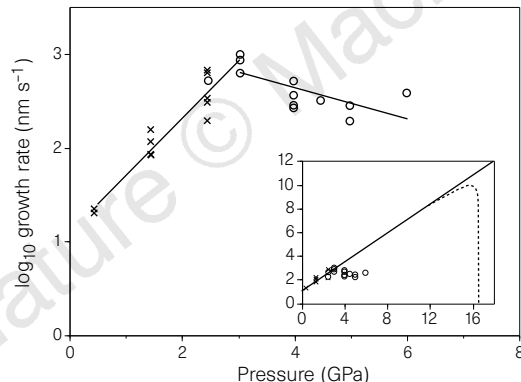


Figure 1 Quartz growth rate at 1,673 K as a function of pressure. Growth rates were measured on Spectrosil W.F., an amorphous silica ($a\text{-SiO}_2$) containing 360 molar p.p.m. Cl and 30 p.p.m. OH. The experimental setup²⁸ and sample preparation procedure² are described elsewhere. Specimens were pressurized at room temperature, heated gradually to 1,073 K and rapidly from 1,073 to 1,673 K, held at constant T for several minutes, quenched rapidly to <473 K, then slowly decompressed at room temperature. Axial sectioning determined the extent of crystal growth. Although coesite is the stable phase above 3 GPa, quartz nucleated on the platinum capsule up to 5 GPa. Above 5 GPa, coesite nucleates unless the sample surface is 'seeded' with finely ground natural quartz. The growth rate is unaffected by the presence of quartz seeds: the measured quartz growth rate from a seeded experiment at 4 GPa coincides with those from unseeded experiments. At 8 GPa, copious coesite nucleation precludes the measurement of a quartz growth rate. Measured growth rates are indicated by crosses (results from ref. 2) and circles (this study). Apparent activation volumes with opposite signs are obtained from the slope of the straight lines fitted to the data below and above 3.0 GPa. Inset: same straight-line fit to low- P data is shown to higher P along with dashed curve from equation (1): ν plunges to zero near 0.086 and 16.4 GPa, where $\Delta G \rightarrow 0$.

over the limited pressure range sampled by their experiment the thermodynamic factor $[1 - \exp(n\Delta G/kT)]$ was constant, thereby attributing all of the change in ν with P to the kinetic, or mobility, factor (D/λ). (They used the lack of curvature in the low- P data to conclude that $n \gg 1$.) Because of its high compressibility, $a\text{-SiO}_2$ is expected to become more stable than quartz at very high pressure¹⁵, whereupon the thermodynamic factor reduces the growth rate to zero. At 1,673 K, $-\Delta G(P)$ calculated by the authors from thermodynamic data in the literature starts at zero near 0 GPa, reaches a maximum of 7 kJ mol^{-1} near 6 GPa, and crosses zero again near 16.4 GPa. Inserting $\Delta G(P)$ into equation (1) and assuming a constant value for $-kT(\partial \ln(D/\lambda)/\partial P)_T$ yields the dashed curve in Fig. 1. Under no combination of thermodynamic uncertainties can the measured behaviour be explained by variations in the final bracketed factor in equation (1); rather than decreasing, this thermodynamic factor increases slightly over the range 3–6 GPa, whereas at 6 GPa ν itself falls below the dashed curve by roughly two orders of magnitude. Hence we must attribute the effect to the mobility factor, (D/λ).

A vanishing migration barrier might occur in several ways. In Fig. 2 we show how it could occur for a mechanism in which crystal growth is caused by the migration of defects at the interface, during which the defect configuration alternates between two states with differing densities. Figure 2a indicates how the standard Gibbs free energy might vary during defect formation and migration. If state C is denser than B then the pressure dependence of these potentials for a pair of adjacent states B and C will be as shown in Fig. 2b. At P_c the migration barrier ($\Delta G_m = G_C^0 - G_B^0$) vanishes although the barrier to defect formation ($\Delta G_f = G_B^0 - G_A^0$) remains nonzero. For $P > P_c$, pressure has so destabilized B that C becomes more stable and a high- P barrier to migration appears as $\Delta G_m^{\text{hip}} = G_B^0 - G_C^0$. At all P the mobility factor in equation (1) is proportional to $\exp -1/kT \times (\max\{G_C^0, G_B^0\} - G_A^0)$, displaying a sharp peak at P_c . This interpretation leads to the interesting prediction of a soft vibrational mode about state B near P_c , perhaps observable with neutron diffraction.

There is growing evidence that network structures tend to have negative activation volumes for atomic transport processes^{1–5,16}. Generally for defect-mediated processes $\Delta V^* = \Delta V_f + \Delta V_m$, with the formation volume $\Delta V_f = \partial \Delta G_f / \partial P$ and the migration volume $\Delta V_m = \partial \Delta G_m / \partial P$. Hence at least one of ΔV_f and ΔV_m tends to be negative, implying a 'free-energy catastrophe' at a critical pressure where a barrier to either formation or migration vanishes. The associated peak in the P -dependence of atomic transport rates may be a general phenomenon for network structures. Although possibly related fundamentally, the observed phenomena leading to the proposed vanishing migration barrier can be contrasted with the shear instability proposed for amorphization^{7,17}. Our observation that quartz and $a\text{-SiO}_2$ continue to exist well past P_c shows that a migration barrier can vanish well before the onset of any shear instability.

This interpretation provides reasonable values of the relevant parameters. The apparent activation volume (with thermodynamic factor roughly constant) for $P < P_c$ is $\Delta V^* = V_c - V_A = \Delta V_f + \Delta V_m$ and for $P > P_c$ is $\Delta V^*, \text{hip} = V_B - V_A = \Delta V_f$, where we define $\Delta V_m = V_c - V_B$ and $\Delta V_f = V_B - V_A$ such that they are always reckoned from the low- P side of P_c . Using the data in Fig. 1, this interpretation yields $\Delta V_m = -24.4 \text{ cm}^3 \text{ mol}^{-1}$ and $\Delta V_f = +5.2 \text{ cm}^3 \text{ mol}^{-1}$. Given a vanishing migration barrier at P_c , we can deduce approximate energies of migration and formation using $G = E + pV - TS$. With no reliable estimates of the entropies of formation and migration, initially we arbitrarily set them to zero (an assumption to be revisited below), resulting in $\Delta E_m \approx -P_c \Delta V_m = 73 \text{ kJ mol}^{-1}$, $\Delta E_f = \Delta E^* - \Delta E_m = 195 \text{ kJ mol}^{-1}$, where we have used Fratello's value² of $\Delta E^* = 268 \text{ kJ mol}^{-1}$.

Fratello *et al.* demonstrated that the presence of trace amounts of OH or Cl is very important in determining the growth rate over the

entire range studied (<10 to 20,000 molar p.p.m.). Similar behaviour has been demonstrated for diffusion in silicates⁴. Everybody's experimental results are likely to have been obtained in the presence of impurity concentrations in this range, as even nominally 'anhydrous' minerals have plenty of OH (ref. 18). Therefore laboratory experimental results may be compared directly to natural processes such as those occurring in the Earth's interior. Molecular dynamics simulations of truly anhydrous material, showing a broad maximum in the diffusivity with increasing pressure¹⁹, may be focusing on a hydrogen-free process that occurs only at much higher temperatures.

A specific example of a mechanism consistent with all of the above observations is the Spaepen–Fratello model^{21,22}, which has been used to account successfully for quartz growth rates below 3 GPa and for the observed solid-phase epitaxial crystallization behaviour of pure amorphous Si (ref. 11). The mechanism involves the thermal rupture of Si–O bonds at Si–O–Si–OH, leading to a sequence of migration events, alternating between O undercoordination (state B) and Si overcoordination (state C). Their proposed mechanism is shown in Fig. 2c; they also showed how multiple migration steps can reconstruct the amorphous network into the crystalline structure before defect annihilation. OH not only can catalyse the rupture but can also give more mobility to the resulting nonbridging O–Si–OH by reducing network constraints. The structural nature of the negative migration volume in this model is evident.

The negative migration volume is very likely due to a transition state (TS) (C in Fig. 2) involving high-coordination Si, but its exact nature is still a matter of debate. It may be due to an overcoordination

state that is sixfold (octahedral) Si (refs 20, 23). Our inferred migration energy and volume are close to the $P = 0$ differences in molar energy and volume between the high-pressure stishovite phase (edge-sharing SiO_6 octahedra) and $\alpha\text{-SiO}_2$ (corner-sharing SiO_4 tetrahedra): they are 1.6 and 1.8 times these differences in molar energy and volume, respectively. So a TS composed of 1–2 SiO_2 units with an energy and volume similar to that of stishovite is quite reasonable. Alternatively, the overcoordination state may be fivefold^{19,21,22,24}, as illustrated in Fig. 2c, because a fivefold coordinated Si state might not be significantly different in energy and volume. Both high-coordination states have been detected in silicate glasses quenched from 6 GPa and above²⁴. However, a coesite-like saddle-point structure (four-membered rings of corner-sharing SiO_4 tetrahedra) is unlikely, as the energy and volume differences with the amorphous phase are too low: to match the inferred migration energy, the TS configuration would have to be composed of ~ 10 SiO_2 units in the coesite structure. Poe *et al.*¹⁶ recently reported a peak in O diffusivity in $\text{Na}_3\text{AlSi}_3\text{O}_{17}$ at 8 GPa and attributed it to a measured²⁴ peak in the concentration of five-coordinated Al. Our measured peak in pure SiO_2 indicates that transitory Si coordination changes cannot be ignored as low as 3 GPa.

In absolute magnitude, the measured growth rate at P_c is consistent with the rate expected from athermal migration of nonbridging oxygen. The thermodynamic factor in equation (1) is about 0.34 near P_c . The site factor f is $C_i \exp(-\Delta G_f/kT)$ where C_i is the mole fraction of catalysing impurity (OH and Cl) at the interface. Assuming no preferential adsorption of impurity at the interface we set C_i to its value in the bulk, $\sim 4 \times 10^{-4}$. From our interpretation of the data, the defect formation enthalpy is $\Delta H_f = \Delta E_f + P_c \Delta V_f = 210 \text{ kJ mol}^{-1}$, resulting in the fraction of Si–O–Si–OH sites that have ruptured at P_c being $\exp(-\Delta G_f/kT) \approx 2.7 \times 10^{-7}$ (assuming the unknown formation entropy is zero), yielding $f = 1.1 \times 10^{-10}$. With a vanishing migration barrier, (D/λ) should attain its maximum value, the velocity of athermal migration, which should be near the speed of sound or $(kT/m)^{1/2}$; both are roughly 1 km s^{-1} . Equation (1) then gives a peak growth rate of roughly 100 nm s^{-1} , to be compared with the experimental value of $1,000 \text{ nm s}^{-1}$. Preferential impurity segregation to the interface and a positive defect formation entropy could easily account for the difference. The latter, in cases where it has been determined (metals and Si), provides multiplicative factors of $10^{1\pm 1}$.

It is perhaps no coincidence that the enthalpy difference between stishovite and $\alpha\text{-SiO}_2$ vanishes near our measured P_c : at 4.7 GPa at our experimental temperature of 1,673 K. Additionally, the pressure of 3.9 GPa at which extrapolations indicate it vanishes at 4,000 K is within the range of pressures for the maximum in oxygen diffusivity against P in the molecular-dynamics simulations of Tsuneyuki and Matsui²⁰. They suggested that the relevant enthalpy difference is between stishovite and coesite, but this difference does not vanish under the conditions of our experiment until 8.4 GPa, three times as far away from the measured P_c .

Why should the difference in H , rather than the difference in G , between the reactant well and the TS vanish near P_c ? According to transition state theory (TST) the activation barrier is in G rather than H . However, in TST the TS is constrained to have one less vibrational degree of freedom than the bulk phases, which implies that although E , V and H might be nearly the same as for bulk phases, the entropy necessarily differs from that of a bulk phase. Furthermore, in classical canonical TST²⁵ there is a Maxwell–Boltzmann distribution of positions and velocities throughout the reactant well (B in Fig. 2a). An increased (decreased) entropy of the TS region (C) of configuration space increases (decreases) the reaction rate. However, as P_c is approached from below and the barrier becomes small enough, escape occurs from the reactant well with incomplete thermalization, reducing the importance of the entropy of the TS region of configuration space. Hence in the small-

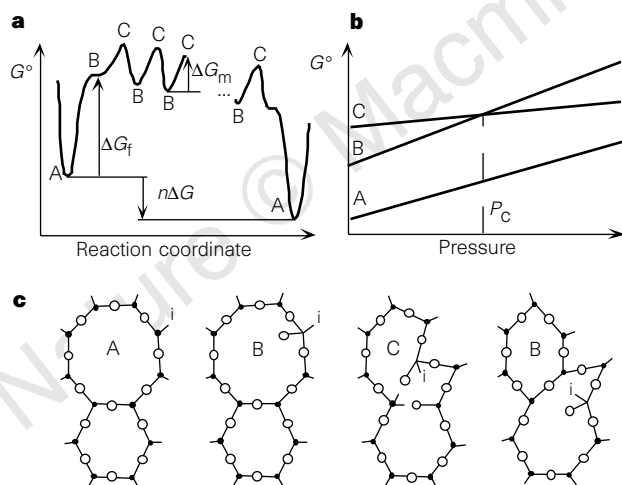


Figure 2 Pressure-induced vanishing of atomic migration barrier owing to 'free-energy catastrophe'. **a**, Standard Gibbs free energy against configuration for three states: A, quartz + $\alpha\text{-SiO}_2$ + interface with no defect; B, same system with defect in high-volume state; C, same system with defect in low-volume state. The 'reaction coordinate' is a path through configuration space along which the reaction proceeds; it is proportional to the number of atoms in the crystal. **b**, Pressure dependence: by the thermodynamic relation $(\partial G/\partial P)_T = V$, the slopes of curves A, B and C are the volumes, V_A , V_B and V_C , of the system in these respective configurations. **c**, Possible migration mechanism, adapted from Fratello *et al.*²² (see also Fig. 17 of Stebbins²⁴), alternating between low-coordination state B (nonbridging O) and high-coordination state C (overcoordinated Si). Si is at each vertex; solid circles represent Si–O bonds normal to the page. A catalysing impurity (OH or Cl) is represented by i. It is reasonable to suppose that the barrier to migration does not entirely vanish, because G° values between points B and C in **a** do not necessarily remain bounded by the values at B and C as P_c is approached. Additionally, near P_c some topography may develop in orthogonal directions revealing new local minima.

barrier limit, the barrier to a reaction might more appropriately be in H rather than in G . This limit is reminiscent of collision theory²⁶. High pressure may thus represent an opportunity for condensed-matter chemical kineticists to investigate systematically the behaviour of kinetic rate constants when kinetic barriers vanish under controllable conditions.

The increase of transport rate in the range 0–3 GPa is very large, implying a significant inverse effect on viscosity of certain rocks and magmas with depth. This is consistent with the experimental work of Kushiro on network structured melts. Our results may be highly relevant to subduction-zone magmatism in the mantle. In this setting partial melting produces magmas enriched in SiO_2 . Although there is little experimental information on the competing effect of temperature on silica-rich melts at high pressure, we propose that such magmas may show a decrease in viscosity with depth until the migration barrier vanishes at 3 GPa (a depth of ~ 100 km), and then suddenly start to become more viscous. We expect that this increase in viscosity may impede silicic magma mobility deeper than 100 km. Interestingly, the predicted viscosity minimum coincides with the nearly constant depth to the Wadati–Benioff zone locating the subduction slab at ~ 100 km beneath most subduction-related volcanic fronts²⁷. This constant depth seems to be largely unaffected by such factors as convergence rate or slab thermal structure. We speculate that the onset of an increasing magma viscosity at a ‘universal’ depth of 100 km, predicted from the present experimental results, may control this aspect of subduction-zone dynamics. There are many unanswered questions such as the effect of SiO_2 content and volatiles on the pressure of the viscosity minimum. The proposed magma viscosity minimum at 3 GPa is a testable hypothesis. Unfortunately, all existing high-pressure experimental data on magma viscosity are limited to 2.5 GPa. Viscosity measurements in the range 2.5 to 5 GPa should be considered a priority. □

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- Kushiro, I. in *Physics of Magmatic Processes* (ed. Hargraves, R. B.) 93–120 (Princeton Univ. Press, Princeton, 1980).
- Fratello, V. J., Hays, J. F. & Turnbull, D. Dependence of growth rate of quartz in fused silica on pressure and impurity content. *J. Appl. Phys.* **51**, 4718–4728 (1980).
- Shimizu, N. & Kushiro, I. Diffusivity of oxygen in jadeite and diopside melts at high pressure. *Geochim. Cosmochim. Acta* **48**, 1295–1303 (1984).
- Goldsmith, J. R. Enhanced Al/Si diffusion in KAlSi_3O_8 at high pressures: the effect of hydrogen. *J. Geol.* **96**, 109–124 (1988).
- Stebbins, J. F., McMillan, P. F. & Dingwell, D. B. (eds) *Structure, Dynamics and Properties of Silicate Melts* (Reviews in Mineralogy Vol. 32, (Mineralogical Society of America, Washington, 1995).
- Williams, Q. & Jeanloz, R. P. Spectroscopic evidence for pressure-induced coordination changes in silicate glasses and melts. *Science* **239**, 902–905 (1988).
- Hemley, R. J., Jephcoat, A. P., Mao, H. K., Ming, L. C. & Manghnani, M. H. Pressure-induced amorphization of crystalline silica. *Nature* **334**, 52–54 (1988).
- Tsuchida, Y. & Yagi, T. New pressure-induced transformations of silica at room temperature. *Nature* **347**, 267–269 (1990).
- Meade, C., Hemley, R. J. & Mao, H. K. High pressure X-ray diffraction of SiO_2 glass. *Phys. Rev. Lett.* **69**, 1387–1390 (1992).
- Nygren, E. *et al.* Pressure dependence of arsenic diffusivity in silicon. *Appl. Phys. Lett.* **47**, 105–107 (1985).
- Lu, G.-Q., Nygren, E. & Aziz, M. J. Pressure-enhanced crystallization kinetics of amorphous Si and Ge: Implications for point defect mechanisms. *J. Appl. Phys.* **70**, 5323–5345 (1991).
- Spaepen, F. & Turnbull, D. in *Laser Annealing of Semiconductors* (eds Poate, J. M. & Mayer, J. W.) 15–42 (Academic, New York, 1982).
- Jackson, K. A., Uhlmann, D. R. & Hunt, J. D. On the nature of crystal growth from the melt. *J. Cryst. Growth* **1**, 1–36 (1967).
- Walker, D., Carpenter, M. A. & Hitch, C. M. Some simplifications to multi-anvil devices for high pressure experiments. *Am. Mineral.* **75**, 1020–1028 (1990).
- Hemley, R. J., Mao, H. K., Bell, P. M. & Mysen, B. O. Raman spectroscopy of SiO_2 glass at high pressure. *Phys. Rev. Lett.* **57**, 747–750 (1986).
- Poe, B. T. *et al.* Silicon and oxygen self-diffusivities in silicate liquids measured to 15 gigapascals and 2800 Kelvin. *Science* **276**, 1245–1248 (1997).
- Tse, J. S. & Klug, D. D. Mechanical instability of α -quartz: a molecular-dynamics study. *Phys. Rev. Lett.* **67**, 3559–3562 (1991).
- Meade, C., Reffner, J. A. & Ito, E. Synchrotron infrared absorbance measurements of hydrogen in MgSiO_3 perovskite. *Science* **264**, 1558–1560 (1994).
- Angell, C. A., Cheeseman, P. A. & Tamaddon, S. Pressure enhancement of ion mobilities in liquid silicates from computer simulation studies to 800 kilobars. *Science* **218**, 885–887 (1982).
- Tsuneyuki, S. & Matsui, Y. Molecular dynamics study of pressure enhancement of ion mobilities in liquid silica. *Phys. Rev. Lett.* **74**, 3197–3200 (1995).
- Spaepen, F. & Turnbull, D. Kinetics of motion of crystal–melt interfaces. *AIP Conf. Proc.* **50**, 73–83 (1979).
- Fratello, V. J., Hays, J. F., Spaepen, F. & Turnbull, D. The mechanism of growth of quartz crystals into fused silica. *J. Appl. Phys.* **51**, 6160–6164 (1980).
- Stolper, E. M. & Ahrens, T. J. On the nature of pressure-induced coordination changes in silicate melts

and glasses. *Geophys. Res. Lett.* **14**, 1231–1233 (1987).

- Stebbins, J. F., in *Structure, Dynamics and Properties of Silicate Melts* (eds Stebbins, J. F., McMillan, P. F. & Dingwell, D. B.) 191–246 (Mineralogical Society of America, Washington, 1995).
- Vineyard, G. H. Frequency factors and isotope effects in solid state rate processes. *J. Phys. Chem. Solids* **3**, 121–127 (1957).
- Laidler, K. J. *Chemical Kinetics* (Harper & Row, New York, 1987).
- Gill, J. *Orogenic Andesites and Plate Tectonics* (Springer, Berlin, 1981).
- Agee, C. B., Li, J., Shannon, M. C. & Circone, S. Pressure–temperature phase diagram for the Allende meteorite. *J. Geophys. Res.* **100**, 17725–17740 (1995).

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Nonlinear ground-motion amplification by sediments during the 1994 Northridge earthquake

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It has been known since at least 1898 (ref. 1) that sediments can amplify earthquake ground motion relative to bedrock. For the weak ground motion accompanying small earthquakes, the amplification due to sediments is well understood in terms of linear elasticity (Hooke's law)², but there has been a long-standing debate regarding the amplification associated with the strong ground motion produced by large earthquakes. The view of geotechnical engineers, based largely on laboratory studies, is that Hooke's law breaks down at larger strains causing a reduced (nonlinear) amplification. Seismologists, on the other hand, have tended to remain sceptical of this nonlinear effect, mainly because the relatively few strong-motion observations seemed to be consistent with linear elasticity. Although some recent earthquake studies have demonstrated nonlinear behaviour under certain circumstances^{3,4}, the significance of nonlinearity for the type of stiff-soil sites found in the greater Los Angeles region remains unresolved⁵. Here we report that ground-motion amplification due to sediments for the main shock of the 1994 Northridge earthquake was up to a factor of two less than the amplification observed for its aftershocks. These observations imply significant nonlinearity in such amplification, and bring into question the use of measurements of weak ground motion to predict the strong ground motion at sedimentary sites.

We compiled data for all locations where both main-shock and aftershock recordings were obtained for the 1994 earthquake. The 21 resultant sites are listed in Table 1 and plotted in Fig. 1. On the basis of surface geology, 15 of these sites are categorized as alluvium (Quaternary sediments), two as soft rock (Tertiary units) and four as relatively hard rock (Mesozoic basement). Also shown in Fig. 1 are the epicentral locations of the 184 aftershocks used in this study, as well as the surface projection of the main-shock rupture distribution. To limit ourselves to a manageable quantity of data, we have used only aftershocks with a magnitude between 3.0 and 5.6.

To estimate site effects in the weak-motion aftershock recordings,

the shear-wave Fourier amplitude spectrum observed at the i th site for the j th event, $O_{ij}(f)$, is represented as

$$O_{ij}(f) = E_j(f) P_{ij}(f) S_i(f) \quad (1)$$

where f is frequency, $E_j(f)$ is the source effect of the j th event, $P_{ij}(f)$ is the path effect for the i th station and j th event, and $S_i(f)$ is the site response for the i th site. The path effect is specified as

$$P_{ij}(f) = r^{-1} e^{-\pi f T_s / Q(f)} \quad (2)$$

where r is the hypocentral distance measured from the aftershock location, T_s is the observed shear-wave travel time, and $Q(f)$ is the quality factor representing attenuation.

The essence of the site-response estimation is as follows. After correcting the observations for the path effect by assuming some $Q(f)$, we estimate the source effects from a site or average of sites, preferably on bedrock, assumed to have no significant site response. The response at the other sites is then estimated as the ratio between the path-corrected observations and the estimated source effects. In practice, however, the entire data set is usually solved simultaneously by some kind of generalized inversion of equation (1)⁶, which provides a convenient mode of book-keeping when not all events are recorded at all sites. Of the many schemes that have been proposed, we follow that of Field and Jacob⁷ to ensure reliable uncertainty estimates.

The main-shock response is estimated as for the aftershocks using equations (1) and (2). However, care must be taken in defining the hypocentral distance, r , because the spatial distribution of rupture (18 by 24 km; ref. 8) can be a significant fraction of the distance to each site. Care should also be taken in defining T_s because the rupture persists for several seconds. Therefore, some kind of average values of r and T_s must be used so that the effects of energy arriving from distances nearer and farther than r , as well as before and after T_s , are averaged out.

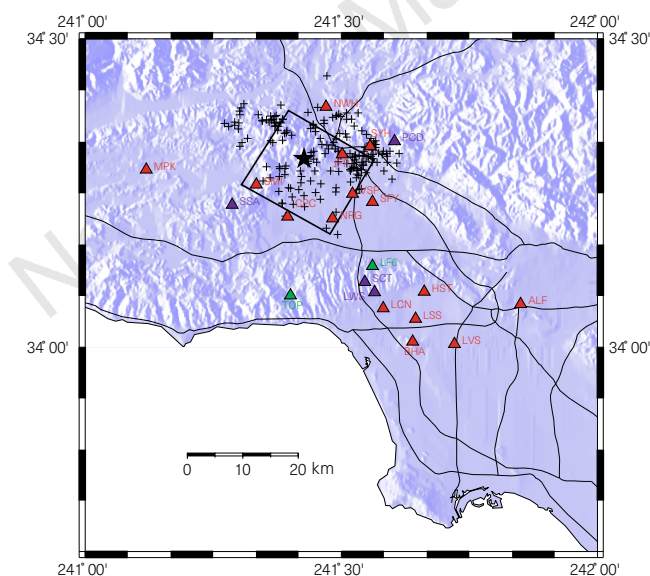


Figure 1 Relief map of the study region. The alluvium recording sites are shown as red triangles, the soft-rock sites as green triangles, and the hard-rock sites as blue triangles. Aftershock epicentres are shown with black crosses, and the main-shock rupture distribution⁸ is outlined by the box. The fault plane dips 40° to the southwest, with the top edge at a depth of 5 km and the bottom edge at a depth of 20.4 km. The location of maximum slip is marked with the black star. All of the main-shock records were obtained from the strong-motion data archive of the Southern California Earthquake Center (SCEC)¹⁷. The aftershock data were obtained from either the United States Geological Survey (USGS)¹⁸ or the SCEC data centre¹⁹.

The estimates of both weak- and strong-motion site response obtained from equations (1) and (2) will be biased by any systematic difference between the actual path effects and those assumed in equation (2), or by any significant site response at the reference site(s). However, because our intention here is to identify any significant differences between the weak- and strong-motion response, and because the travel paths are similar for the main shock and aftershocks, these sources of bias will not influence the comparison as long as the same $Q(f)$ and reference-site definition are applied in both cases.

One additional complication is finite-source effects, such as directivity^{9,10}, in the strong-motion observations. These result from the large spatial extent of the main-shock rupture, where energy arriving from different locations on the fault plane may interfere constructively or destructively causing $E_j(f)$ to vary with site location. Therefore, appropriate spatial averages must be taken, or corrections applied, to avoid such biases.

Figure 2 shows the weak- and strong-motion site-response estimates averaged over the 15 quaternary alluvium sites. These estimates were computed relative to the average of the four hard-rock sites, and we followed Hartzell¹¹ in assuming $Q(f) = 150 f^{1/2}$. The weak-motion response implies an amplification factor of ~ 3.1 at 1 Hz, decreasing to factors of ~ 2.5 and ~ 1.4 at 3 and 10 Hz, respectively. The strong-motion amplification factors are systematically less, being ~ 1.9 at 1 Hz, ~ 1.3 at 3 Hz, and ~ 0.8 (deamplification) at 10 Hz. This lower amplification for the main shock implies nonlinearity.

To test the null hypothesis that sediment amplification was similar for the main shock and the aftershocks, Fig. 3 shows the ratios of the weak- to strong-motion estimates at each sediment site. Also shown are the mean and 95% confidence region assuming a log-normal distribution⁷. The difference from unity is significant over almost the entire frequency band, and between 0.8 and 5.5 Hz at the 99% confidence level, leading to the rejection of the null hypothesis. In other words, sediment amplification seems to have been significantly less during the main shock, implying nonlinearity.

The difference as plotted in Fig. 3 is greatest between 2 and 4 Hz. For reference, the values of the individual ratios at 3 Hz are listed for each site in Table 1. One might ask whether the difference depends

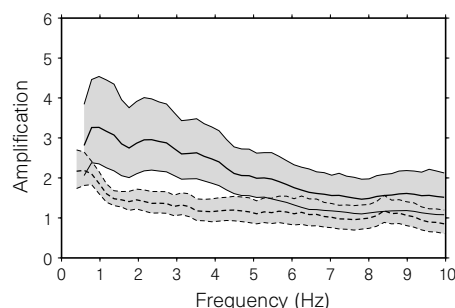


Figure 2 The mean and ± 2 standard-deviation-of-the-mean confidence limits of the estimates of amplification at the 15 alluvium sites. The solid lines represent the weak-motion results for the aftershocks, and the dashed lines represent the strong-motion results for the main shock. The lower-frequency cutoffs reflect the lowest resolvable frequencies given seismometer and noise limitations. The Fourier amplitude spectra were computed from 20-s windows starting 1 s before the S-wave arrival, and were smoothed with a boxcar function that increased in width from 0.5 to 1.3 Hz between 0.5 and 10 Hz. The results presented here, however, do not depend on this particular spectral estimation technique. A stringent data elimination scheme was used to minimize the effects of noise⁷, and the two horizontal components were geometrically averaged. Furthermore, to minimize the effects of location uncertainties, weak-motion recordings of aftershocks closer than 10 km were eliminated.

on the two highest ratios in Fig. 3 (VSP and JFP). In fact, at 3 Hz the highest 10 ratios (out of 15) must be removed from the average before the difference becomes insignificant at the 95% confidence level.

Many tests have been conducted to evaluate the robustness of this observation (E.H.F., Y.Z., P.A.J. and I.A.B., manuscript in preparation). As mentioned previously, any shortcomings of equation (2) in representing the path effects will be mapped onto the weak- and strong-motion estimates similarly, and therefore should not influence the ratios in Fig. 3. Nevertheless, we applied the *Q* models of Peng¹² and of Bonillia *et al.*¹³, as well as a constant *Q* of 328, and found the results unchanged. Although bedrock sites can show their own unique behaviour^{14,15}, any significant site effect in our reference-site definition should influence both the weak- and strong-motion estimates similarly. Indeed, although the amplification levels shown in Fig. 2 depend on the reference definition, the significant difference shown in Fig. 3 does not depend on the inclusion of any particular rock site.

By far the most problematic source of bias could result from the large spatial extent of rupture during the main shock. For the strong-motion estimates *r* and *T_s* were determined from the location and timing of maximum slip as determined by the inversion of Wald *et al.*⁸. This point, shown as a star in Fig. 1, ruptured about 4.5 seconds after slip initiation at the hypocentre. However, we also

computed *r* from all four corners of the rupture plane, and *T_s* from both the rupture initiation and termination 7 seconds later, and found the conclusions regarding the null hypothesis unchanged.

As an additional test of whether finite-source effects for the closest sites might be biasing the result, we re-did the analysis using only the more distant sites in the Los Angeles basin (LCN, HST, LSS, BHA, LVS and ALF) relative to the rock site SCT. Although not as pronounced, the difference still persists. For example, at 3 Hz the difference is a factor of 1.6 and is significant at the 95% confidence level. That the difference is lower is consistent with the notion that nonlinearity at the more distant sites, where ground motion levels are lower, will be less.

As a final test of whether finite-source effects might be masquerading as nonlinearity, we computed synthetic seismograms using the methodology and Northridge rupture model of Zeng and Anderson¹⁶. Specifically, for the 21 sites considered here, and using the one-dimensional velocity model given in their Table 2, we computed synthetic seismograms for the main shock and nine relatively small events distributed equally over the main-shock rupture plane. By conducting an analysis identical to that used to generate Figs 2 and 3, we have found no evidence for a significant bias due to finite source effects.

To infer the presence of nonlinear response amongst the myriad of competing effects, we have been forced to combine the results from numerous alluvium sites. However, by such averaging we sacrifice physical insight into the nature of the nonlinearity. Having convinced ourselves that the nonlinearity exists, we can now examine individual sites more carefully to detect shifts in resonant frequencies and/or reductions in amplification factors as a function of input amplitudes, both of which are symptomatic of nonlinear response. A sediment resonance that is clear in the weak-motion site-response estimate (for four of the sites, SMI, NWH, JFP and LF6) is conspicuous by its absence in the strong-motion results (again implying nonlinearity). Such observations will help test the validity of the laboratory-based methodologies used by engineers, and will provide a basis for establishing equation-of-state relationships for use in theoretical computations.

Finally, the conclusion of significant nonlinearity is good news in that the amplifying effects of sediments, on average, are apparently not as great as implied by weak-motion studies. However, it brings into question the use of empirical Green's functions (based on recordings of small earthquakes) to study or predict strong ground motion at sediment sites. □

Table 1 The 21 locations in the study region

Site name	Site geology*	Latitude	Longitude	Number of aftershocks	Ratio at 3 Hz†	PGA‡ (cm s ⁻¹)
CPC	alluvium	34.212	-118.605	53	1.77	538
JFP	alluvium	34.313	-118.498	32	7.98	616
LCN	alluvium	34.063	-118.418	57	1.99	251
HST	alluvium	34.090	-118.338	91	1.40	225
ALF	alluvium	34.070	-118.150	69	1.27	99
MPK	alluvium	34.288	-118.881	26	1.52	286
NWH	alluvium	34.390	-118.530	42	2.05	578
SFY	alluvium	34.236	-118.439	44	2.90	541
VSP	alluvium	34.249	-118.478	5	9.37	923
LVS	alluvium	34.005	-118.279	6	0.91	285
LSS	alluvium	34.046	-118.355	2	2.52	482
SYH	alluvium	34.326	-118.444	19	1.60	827
BHA	alluvium	34.009	-118.361	15	1.83	234
NRG	alluvium	34.209	-118.517	9	2.28	468
SMI	alluvium	34.264	-118.666	36	1.28	924
LF6	soft rock	34.132	-118.439	85	5.96	498
TOP	soft rock	34.084	-118.599	4	1.32	327
SSA	hard rock	34.231	-118.713	26	1.52	336
SCT	hard rock	34.106	-118.454	184	0.97	371
PCD	hard rock	34.334	-118.396	25	0.42	426
LWS	hard rock	34.089	-118.435	14	1.61	293

* As defined in the SCEC strong-motion data archive¹⁷.

† The ratio of weak- to strong-motion site response estimate at 3 Hz.

‡ Peak ground acceleration observed during the Northridge main shock¹⁷.

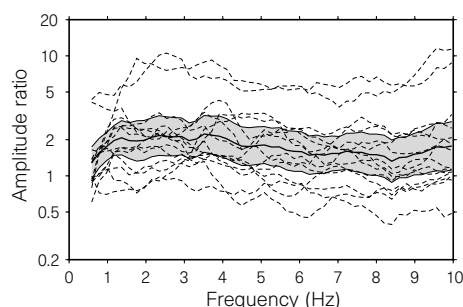


Figure 3 Ratios of the estimates of weak- to strong-motion site responses for each of the 15 sediment sites (dashed lines). The values at 3 Hz are listed in Table 1. The mean and 95% confidence region of all the sediment-site ratios are plotted with the solid lines and shaded region (based on a *t*-distribution with 14 degrees of freedom). The weak-motion amplification estimates are, on average, significantly higher, implying nonlinearity.

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- Milne, J. *Seismology* 1st edn (Kegan Paul, Trench, Truber, London, 1898).
- Aki, K. Local site effects on strong ground motion. *Proc. Earthq. Eng. Soil Dyn.* **II**, 103–155 (1988).
- Bersenev, I. A. & Wen, K.-L. Nonlinear soil response—A reality? *Bull. Seismol. Soc. Am.* **86**, 1964–1978 (1997).
- Aguirre, J. & Irikura, K. Preliminary analysis of non-linear site effects at Port Island array station during the 1995 Hyogoken–Nambu earthquake. *J. Nat. Dis. Sci.* **16**, 49–58 (1995).
- Petersen, M. D. *et al.* Seismic ground-motion hazard mapping incorporating site effects for Los Angeles, Orange, and Ventura counties. *Bull. Seismol. Soc. Am.* **87**, 249–255 (1997).
- Andrews, D. J. Objective determination of source parameters and similarity of earthquakes of different size. *Earthq. Source Mech. Geophys. Monogr.* **37**, 6, 259–268 (1986).
- Field, E. H. & Jacob, K. H. A comparison and test of various site response estimation techniques, including three that are not reference site dependent. *Bull. Seismol. Soc. Am.* **85**, 1127–1143 (1995).
- Wald, D. J. *et al.* The slip history of the 1994 Northridge, California earthquake determined from strong-motion, teleseismic, GPS, and leveling data. *Bull. Seismol. Soc. Am.* **86**, S49–S70 (1996).
- Archuleta, R. J. & Hartzell, S. H. Effects of fault finiteness on near-source ground motion. *Bull. Seismol. Soc. Am.* **71**, 939–957 (1981).
- Heaton, T. H. Evidence for and implications of self-healing pulses of slip in earthquake rupture. *Phys. Earth Planet. Inter.* **64**, 1–20 (1990).
- Hartzell, S. *et al.* Site response for urban Los Angeles using aftershocks of the Northridge earthquake. *Bull. Seismol. Soc. Am.* **86**, S168–S192 (1996).
- Peng, J. Y. Spatial and temporal variation of coda *Q* in California. Thesis, Univ. South California, Los Angeles (1989).
- Bonilla, L. F. *et al.* Site amplification in the San Fernando Valley, CA: Variability of site effect estimation using the S-wave, coda, and H/V methods. *Bull. Seismol. Soc. Am.* **87**, 710–730 (1997).
- Cranswick, E. The information content of high-frequency seismograms and the near-surface geologic structure of “hard rock” recording sites. *Pure Appl. Geophys.* **128**, 333–363 (1988).
- Steidl, J. *et al.* What is a reference site? *Bull. Seismol. Soc. Am.* **86**, 1733–1748 (1996).
- Zeng, Y. & Anderson, J. G. A composite source model of the 1994 Northridge earthquake using genetic algorithms. *Bull. Seismol. Soc. Am.* **86**, S71–S83 (1996).
- Strong-motion data archive of the Southern California Earthquake Center (web address: <http://quake.crustal.ucsb.edu/scec/smdb/smdb.html>).

18. Meremonte, M. *et al.* Urban seismology—Northridge after shocks recorded by multiscale arrays of portable digital seismographs. *Bull Seismol. Soc. Am.* **86**, 1350–1363 (1996).
19. Data Center of the Southern California Earthquake Center (web address: <http://www.sceec.edu>).

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Absence of contour linking in peripheral vision

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Human foveal vision is subserved initially by groups of spatial, temporal and orientational 'filters', the outputs of which are combined to define perceptual objects. Although a great deal is known about the filtering properties of individual cortical cells, relatively little is known about the nature of this 'linking' process. One recent approach¹ has shown that the process can be thought of in terms of an association field whose strength is determined conjointly by the orientation and distance of the object. Here we describe a fundamental difference in this feature-linking process in central and peripheral parts of the visual field, which provides insight into the ways that foveal and peripheral visual perception differ^{2,3}. In the fovea, performance can be explained only by intercellular linking operations whereas in the periphery intracellular filtering will suffice. This difference represents a substantial economy in cortical neuronal processing of peripheral visual information and may allow a recent theory of intercellular binding to be tested^{4–7}.

The way that distributed neuronal activity in the cortex is combined to define perceptual objects is an important question in neurobiology⁷. In human vision, one promising method of study involves the detection of paths of spatially narrowband elements embedded in a field of similar elements with random position and orientation¹. The elements forming the path differ from those of the background in that they conform to first-order curves (Fig. 1). Human detection performance is nearly perfect for paths with

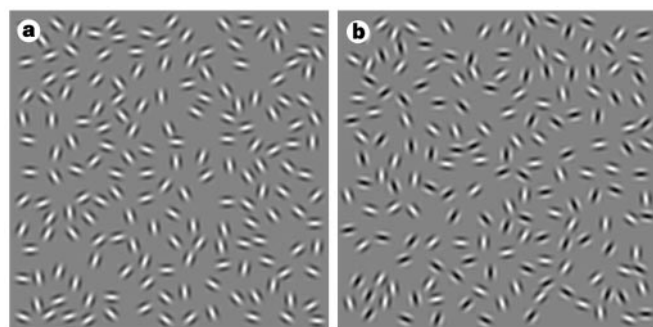


Figure 1 An example of the path stimuli used. **a**, All elements are localized in spatial frequency and are of cosine phase. The path, which is slightly curved, runs from bottom to top through the middle of the picture. **b**, A similar path, but now composed of similar elements alternating in spatial phase by 180°, curves from the bottom left to the top right, embedded in a background field of alternating-phase elements. In the fovea both types of path are easily detected, but in the periphery, the path shown in **b** is invisible.

orientation differences between neighbouring elements of up to 20–30 degrees (Fig. 1a). It has been claimed¹, but never substantiated, that such performance could not be supported solely by individual cortical filters, and hence requires an integrative or linking process between cells analysing different orientations. We find that this claim is indeed correct.

We used a path-detection model operating on multiple, independent oriented filter outputs (Fig. 2 and Methods). The image is initially orientation-filtered at a scale appropriate to the elements. After thresholding, a symbolic description of the resulting orientation features is computed (a two-dimensional adaptation of the MIRAGE algorithm⁸). The path is then indicated by the longest feature present across all orientations. In the example in Fig. 2 the path angle is zero and filters aligned with the straight path correctly encode the path perceived in Fig. 2.

The solid line in Fig. 3a represents the output of our filter model, compared with the performance of human foveal vision. Model performance is much worse than that of human subjects when path angle is increased and when the paths become more curved (Fig. 3a). We could find no method of improving the performance of the model without introducing interactions between cells tuned to different orientations, providing a quantitative demonstration that filtering alone (intracellular processing), at any one of a number of orientations, is insufficient to explain human performance. A direct test of this conclusion is to use paths composed of elements whose spatial phases are alternately switched by 180°, embedded in a background of elements with spatial phase set randomly to either 0° or 180°. Such a stimulus (Fig. 1b), which cannot be detected by a simple linear filtering operation (model results are shown as a solid

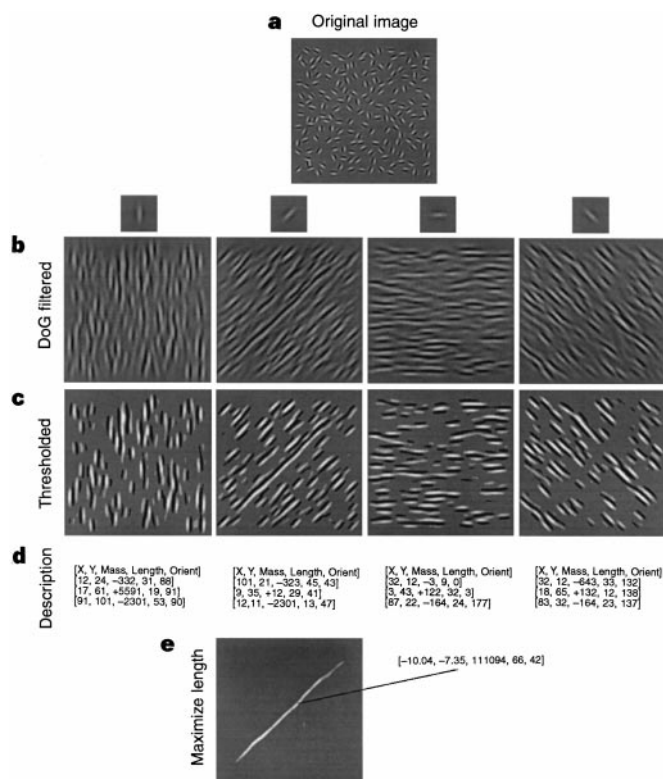


Figure 2 The simple-filter model. **a**, An example image. **b**, The operation of four filters from the full bank of 12 is shown. **c**, These filter outputs are 'thresholded' (all grey levels falling within ± 1 s.d. of the mean are replaced with the mean value), producing a new image containing both positive and negative polarity 'blobs' (here blobs have been contrast-enhanced to demarcate them further). **d**, Descriptions of the 'blobs' in each of these images, by which it is possible to identify the longest blob across all filter outputs, identified as the 'path'.

line in Fig. 3b) results in only a modest decrement of performance for human foveal vision (filled symbols in Fig. 3b), adding weight to the suggestion that linking processes between cells tuned to different orientations are necessary to explain foveal contour integration.

Evaluation of peripheral visual function with the same stimuli produces a very different conclusion. Figure 3c shows results for a range of eccentric loci in the visual field (10°, 20°, 25°, and 30° into the temporal retina). Path detection is constant over a wide range of viewing distances (the task exhibits scale invariance) as seen from the correspondence of data when using stimuli differing in scale by a factor of eight (Fig. 3c). This obviates the need to scale stimuli to allow for known variation in cortical magnification across the visual field. Furthermore, by using an orientation-discrimination task we ensured that the carrier frequency of our patterns was below the Nyquist limit at the largest eccentricity tested⁹. Our results reveal not only a reduction in peripheral performance for larger path angles but also invariant performance beyond about 10° eccentricity. The known differences between fovea and periphery cannot account for the latter finding, but our model of the filtering properties of individual cortical cells (Fig. 3c) is sufficient to explain peripheral performance. Furthermore, beyond 10° eccentricity, paths composed of elements with randomly alternating phase are undetectable

(Fig. 3d), indicating that peripheral paths might be seen using no more than filtering operations (solid line in Fig. 3d shows predictions of such a model). This conclusion is also supported by subjects' reported perception that peripheral paths, unlike their foveal counterparts, appear perceptually smeared along their length (sometimes described by subjects as 'creases').

The need for substantial interaction between cells tuned to different orientations to integrate the distributed neuronal activity for the definition of simple contours carries with it a substantial commitment of cortical resources. Our finding indicates that the human visual system might limit such interactions to central vision. In the periphery, the visual system relies more on simple filtering operations at independent orientations to define contours, representing a substantial economy in cortical processing. It has long been argued that foveal and peripheral processing occur along quite different lines^{2,3}, the former involving discrimination, the latter detection. But to date, the only quantitative differences found between foveal and peripheral visual processing have involved contrast thresholds and spatial scale, neither of which could support such a claim. A difference in the way contours are defined could form the computational basis of such a claim. Recent attempts to understand the physiological basis of contour binding have implicated the long-range cortical interactions observed anatomically¹⁰ and physiologically¹¹, and the synchronization of cortical oscillations observed physiologically⁴⁻⁷. Whatever the physiological basis, in the light of our results, it should be limited to the central visual field.

Methods

Psychophysics. Paths had a backbone of eight invisible line segments; each line segment was of the same length and joined other segments at an angle uniformly distributed between $+\alpha$ and $-\alpha$, where α is the path angle¹ and varies from $\alpha = 0^\circ$ (straight) to $\alpha = 40^\circ$ (very curved). Subjects were shown two images in random order on each of 100 presentations, one containing just the background elements and another containing a path embedded in a field of background elements. Element density was the same in these two presentations. Subjects were asked to select the presentation containing the path. The elements were narrowband in spatial frequency and orientation, and were displayed at a contrast of 90%, all being in sine phase for a duration of 1 s. The carrier of the Gabor patches was set to 3 cycles per degree, the gaussian envelope had $\sigma = 8$ arc min. and the inter-element spacing as 3.35 times the carrier period.

Computational model. Images were initially convolved with a bank of 12 spatial filters oriented from 0° – 165° in steps of 15° . Filters had a peak spatial frequency matched to the carrier component of the Gabor patches comprising the stimulus. As long as the filter is band-limited in the spatial-frequency and orientation domains, its exact form is unimportant. We used two-dimensional difference-of-gaussian (DoG) filters composed of a DoG in the x direction multiplied by a gaussian function in the y direction:

$$W(x, y) = \left[\exp(-x_i^2/2\sigma^2) - \frac{1}{1.23} \exp(-x_i^2/2(2.23\sigma)^2) \right] \exp(-y_i^2/2(3\sigma)^2)$$

where σ refers to the standard deviation of the positive gaussian function and (x_i, y_i) are coordinates rotated by angle ϕ :

$$x_i = x \cos \phi + y \sin \phi$$

$$y_i = y \cos \phi - x \sin \phi$$

Parameters of the DoG are based on those derived in ref. 12.

Filter output is 'thresholded' by setting all grey levels falling within ± 1 standard deviation of the mean to the mean value itself. The thresholding operation serves to reduce the effect of noise and to avoid production of very large or complex two-dimensional 'blobs'. A symbolic description of each of these 'blobs' is generated as a list of parameters describing the position, orientation, length, width, and so on¹³. By selecting the longest blob across all filter orientations, embedded contours may be detected (if orientation differences between adjacent components are sufficiently small). To compare the predictions of this model to human data, we used a procedure for generating

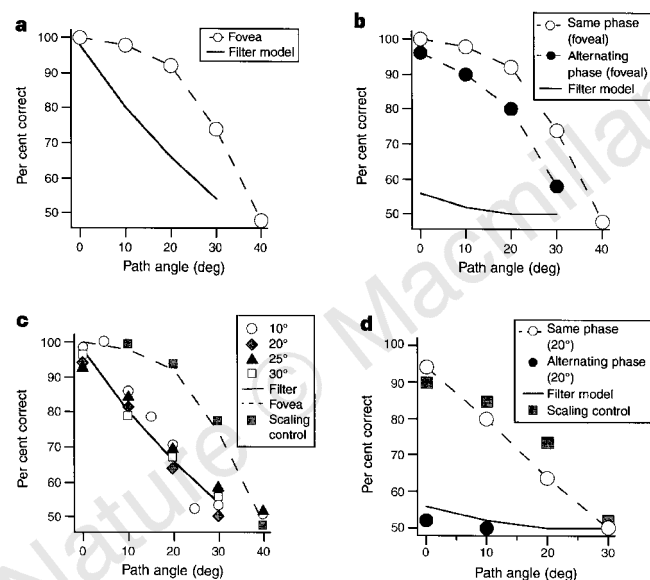


Figure 3 Detection of paths embedded in a background field of randomly oriented elements. Performance (% correct choices) is plotted as a function of path angle. In each frame human performance is compared with the performance of the 'simple-filter' model in which there is no integration across filters tuned to different orientations. **a**, Foveal performance is compared with that of the simple-filter model. **b**, Foveal performance is compared for elements with alternating spatial phase with that of the filtering model. For comparison human performance for elements with the same spatial phase is shown. **c**, Human performance (with the same stimuli as in **a**) for a range of eccentric loci in the visual field (10°, 20°, 25°, 30°) is compared with the predictions of the filtering model. Foveal performance is shown at two different scales (dashed line: carrier is 3 cycles per degree (c.p.d.); shaded squares: carrier is 24 c.p.d.), demonstrating scale invariance, so decreased performance in the periphery cannot be due to its coarser neural sampling. **d**, Peripheral performance for 20° eccentricity is compared for elements with alternating spatial phase with that of the filtering model. For comparison human performance at the 20° locus for elements having the same spatial phase is shown. Squares indicate foveal performance with an alternating phase path which has been scaled (carrier is 24 c.p.d.) to reflect the decrease in neural sampling at 20° eccentricity. Poor performance with alternating phase paths in the periphery is clearly not attributable to a scaling difference between fovea and periphery.

stimuli similar to that used for the psychophysical experiment. Pairs of textures either containing or not containing an embedded contour were generated and processed using the model. The longest blob was calculated for both images, and the image producing the longer of the two was selected as the one containing the contour. Performance of the model was determined as a function of path angle (the orientational difference between successive elements of the contour).

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- Field, D. J., Hayes, A. & Hess, R. F. Contour integration by the human visual system: evidence for a local "association field". *Vision Res.* **33**, 173–193 (1993).
- Aubert, H. & Foerster, E. Unter suchungen über raumsinn der retina. *Albrecht v. Graefes Arch. Ophthalmol.* **3**, 1–37 (1857).
- Schneider, G. E. Two visual systems. *Science* **163**, 895–902 (1969).
- von der Malsburg, C. in *Synergetics of the Brain* (ed. Basar, E., Flohr, H., Haken, H. & Mandall, A. J.) 238–249 (Springer, Berlin, 1983).
- von der Malsburg, C. & Singer, W. in *Neurobiology of Neocortex* (eds Rakic, P. & Singer, W.) 69–99 (Wiley, Chichester, 1988).
- Kreiter, A. K. & Singer, W. Global stimulus arrangement determines synchronization of neuronal activity in the awake macaque monkey. *Eur. J. Neurosci.* (suppl.) **7**, 153 (1994).
- Engel, A. K., König, P., Kreiter, A. K., Schillen, T. B. & Singer, W. Temporal coding in the visual cortex: new vistas on integration in the nervous system. *Trends Neurosci.* **15**, 218–226 (1992).
- Watt, R. J. & Morgan, M. J. A theory of the primitive spatial code in human vision. *Vis. Res.* **25**, 1661–1674 (1985).
- Wang, Y., Thibos, L. N. & Bradley, A. Undersampling produces non-veridical motion perception, but not necessarily motion reversal, in peripheral vision. *Vision Res.* **36**, 1737–1744 (1996).
- Gilbert, C. D. & Wiesel, T. N. Columnar specificity of intrinsic horizontal and corticocortical connections in cat visual cortex. *J. Neurosci.* **9**, 2432–2442 (1989).
- Tso, D. Y. & Gilbert, C. D. The organization of chromatic and spatial interactions in the primate striate cortex. *J. Neurosci.* **8**, 1712–1727 (1988).
- Phillips, G. & Wilson, H. Orientation bandwidths of spatial mechanisms measured by masking. *J. Opt. Soc. Am.* **62**, 226–232 (1983).
- Watt, R. J. *Understanding Vision* (Academic, London, 1991).

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Fear conditioning induces associative long-term potentiation in the amygdala

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Long-term potentiation (LTP) is an experience-dependent form of neural plasticity believed to involve mechanisms that underlie memory formation^{1–3}. LTP has been studied most extensively in the hippocampus, but the relation between hippocampal LTP and memory has been difficult to establish^{4–6}. Here we explore the relation between LTP and memory in fear conditioning, an amygdala-dependent form of learning in which an innocuous conditioned stimulus (CS) elicits fear responses after being associatively paired with an aversive unconditioned stimulus (US). We have previously shown that LTP induction in pathways that transmit auditory CS information to the lateral nucleus of the amygdala (LA) increases auditory-evoked field potentials in this nucleus⁷. Now we show that fear conditioning alters auditory CS-evoked responses in LA in the same way as LTP induction. The changes parallel the acquisition of CS-elicited fear behaviour, are enduring, and do not occur if the CS and US remain unpaired. LTP-like associative processes thus occur during fear conditioning, and these may underlie the long-term associative plasticity that constitutes memory of the conditioning experience.

To determine whether fear conditioning results in learning-related changes in CS processing that are similar to the effect of LTP induction in auditory CS pathways, we concurrently measured auditory CS-evoked field potentials in LA and CS-evoked fear behaviour, before, during and after fear conditioning in freely behaving rats. The rats were randomly assigned to groups that underwent either fear conditioning (in which the CS and US were

paired) or a non-associative control procedure (in which the CS and US were explicitly unpaired). The CS was a 20-s series of acoustic tones (1 kHz, 50 ms, 72 dB) delivered at 1 Hz. The onset of each tone in the series triggered the acquisition of an evoked waveform from the electrode in LA, so that each 20-s CS produced 20 evoked responses. The 100 evoked waveforms from each session (5 CS per session; mean inter-CS interval, 170 s, range 140–200 s) were averaged to yield a mean CS-evoked field potential (CS-EP) for that session. The use of this 'one tone per second' 20-s CS allowed the sampling of CS-evoked activity at 20 points within a single CS, greatly increasing the signal-to-noise ratio of the field potentials under study over that obtainable with the continuous-tone CS typically used in conditioning studies^{8–10}.

The CS-EPs were quantified by measuring the latency, slope and amplitude of the negative-going potential occurring 15–30 ms after the onset of the tone stimulus, as per our previous study of auditory evoked field potentials in LA⁷. Anatomical and physiological evidence indicates that these field potentials are generated in the LA⁶. A set of CS-EPs for two rats, one from the 'conditioned' group and one from the 'control' group, over the seven sessions of testing and training is shown in Fig. 1a. As previously reported⁷, before training the CS elicited a negative-going field potential with a latency of about 18 ms (18.52 ± 3.58 ms across animals). The raw (not normalized) slope and amplitude of these potentials did not differ between the two groups in the baseline tests before training (slope: conditioned group, $-1.649 \pm .425 \mu\text{V ms}^{-1}$, control group, $-2.329 \pm .346 \mu\text{V ms}^{-1}$; *t*-test, *P* > 0.05, conditioned group, $14.186 \pm 4.103 \mu\text{V}$; control group, 18.116 ± 4.214 ; *t*-test, *P* > 0.05). As seen in the examples shown (Fig. 1a), paired training led to an increase in the slope and amplitude of the CS-EPs, whereas unpaired training did not. Mean group data of slope and amplitude of CS-EPs, normalized as a percentage of mean baseline measures, are shown in Fig. 1b. For both groups, slope and amplitude were stable for the first two sessions (testing), in which only the CS was presented. Responses in these sessions were used as a baseline from which to measure changes due to training. For the conditioned group, slope and amplitude were unchanged by unpaired presentations of the CS and US in session 3, but increased significantly above baseline in sessions 4 and 5 when the CS was paired with the US (statistics in Fig. 1b). Both measures remained elevated in session 6, in which only the CS was presented, and fell towards baseline in the last session, reflecting the weakening of the CS-US relation by presentations of the CS without the US (extinction trials). The slope and amplitude of the CS-EPs remained statistically unchanged throughout the course of training and testing for the control group (statistics in Fig. 1b). Slope and amplitude did not differ between the groups until pairing occurred, and remained different until the last session (statistics in Fig. 1b). The fact that the two groups received an equal number of CS and US presentations during training, and that unpaired training was not accompanied by increases in CS-EPs in either group, indicates that the effect of paired training on the field potentials in the conditioned group is due to the associative relation of the CS and US and not to nonspecific arousal elicited by either stimulus alone¹¹.

The differential effects of training on CS-EPs for each member of the control and conditioned groups is shown in Fig. 2. This scattergram demonstrates the consistency with which the control group was unaffected by training and the reliability of the increases in slope and amplitude of the CS-EPs in the conditioned group.

The acquisition of conditioned fear behaviour was evaluated by measuring 'freezing', a characteristic defensive posture expressed in the presence of stimuli that predict danger^{12–15}. The amount of time accounted for by freezing was measured during the 20-s CS and also during the 20 s immediately before CS onset (pre-CS period). The latter is a measure of the acquisition of aversive conditioning to the experimental context in which the US is delivered (such as the conditioning chamber); freezing to the experimental context is

independent of the presence or absence of an explicit CS, and is typically seen with both paired and unpaired training^{8,10}. In this experiment and pilot studies, the pattern of behaviour exhibited during the 'one tone per second' 20-s CS was in all respects similar to the behaviour exhibited by animals trained with a 20-s continuous tone CS; for example, rats did not respond to the individual tones that made up the CS, but rather behaved as though the 20-s CS period was a continuous tone.

Analyses of variance and post hoc tests of the behavioural data showed the expected result from paired and unpaired training. Thus there was a significant interaction between group and session, owing to the higher level of freezing in the conditioned group in session 6, the first test session after training (statistics in Fig. 1c). This was also

the session in which the CS-EP measures differed most between the groups (Fig. 1b). By the next session, freezing responses, like field-potential measures, no longer differed between the groups, showing that the CS-US relation had extinguished. Although both groups froze extensively during training (sessions 3–5), freezing measured in sessions with US presentations is not generally useful as an index of CS-related learning, owing to the confounding effects of the US on freezing behaviour⁸.

To further investigate the differential effect of paired versus unpaired training we analysed freezing before the CS and during the CS in the two groups (data not shown). Pre-CS freezing, which reflects conditioning to contextual stimuli⁸, did not differ between the conditioned and control groups at any point in the course of

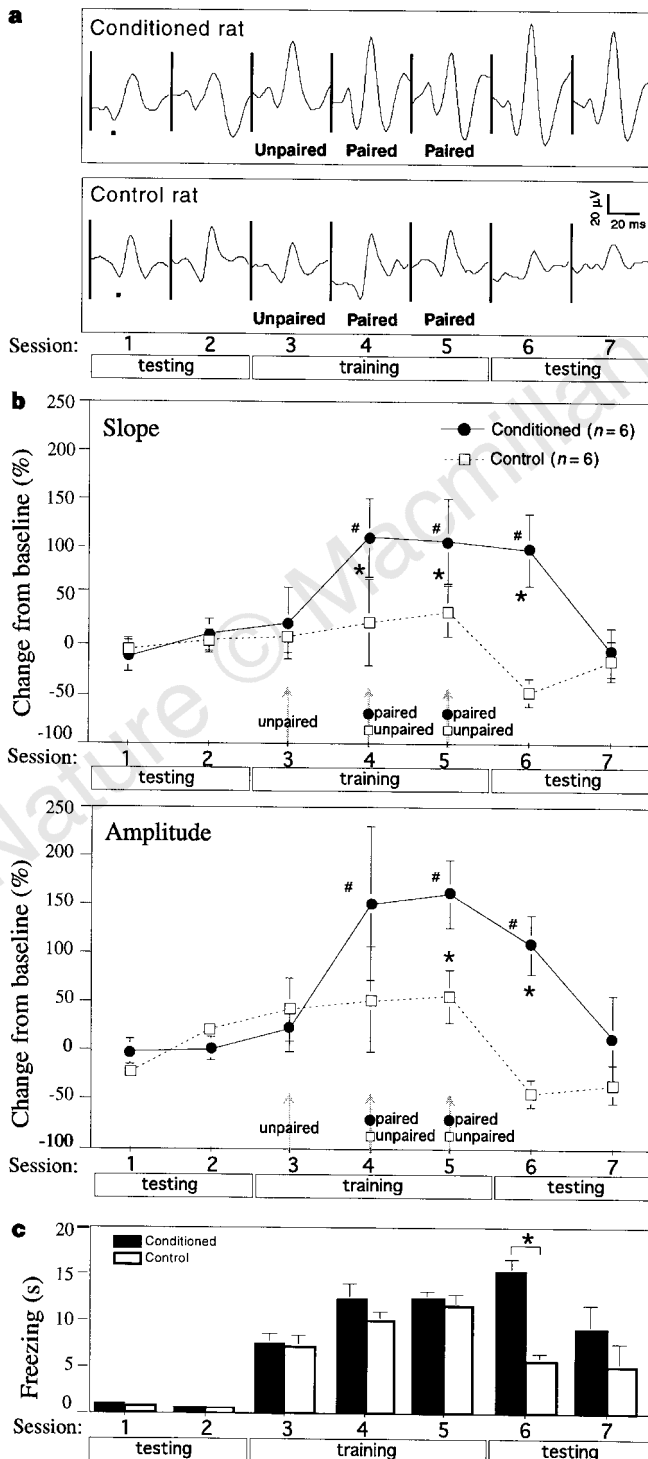


Figure 1 The effect of paired and unpaired training on CS-evoked field potentials and behaviour. Sessions are numbered 1–7; one session occurred per day, except that sessions 3 and 4 occurred on the same day. **a**, CS-evoked field potentials from a conditioned rat (top) and a control rat (bottom), covering the full-time course of the experiment. Quantitative analysis was performed on the first negative (downward)-going deflection (dot). Our previous studies of these waveforms have concentrated on this feature as it has the shortest latency, is reliably present, coincides with local evoked unit activity, shows experience-dependent plasticity, and reflects transmission from the auditory thalamus to the amygdala^{7,20}. The other components of the waveform visible in these examples are not reliably present across trials and subjects, and little is known about their origin and mechanisms⁷. **b**, Fear conditioning increases the slope and amplitude of CS-EPs, but unpaired training does not. Slope and amplitude of the negative-going potential are normalized as a percentage of the mean values before training (sessions 1 and 2). The normalized slope and amplitude of the evoked potentials were evaluated statistically with two-factor ANOVAs with group (conditioned, control) as the between-subjects factor and experimental session as within-subject factor. A significant group–session interaction was observed for both measures (slope, $F(6, 60) = 2.59$, $P < 0.05$; amplitude, $F(6, 60) = 2.70$; $P < 0.05$). Significant differences of post hoc analyses are indicated (*Duncan, $P < 0.05$, between groups; #Duncan, $P < 0.05$, within group with respect to pretraining sessions 1 and 2). Error bars, $\pm$ s.e.m. **c**, Fear conditioning leads to associative conditioning of fear behaviour. Freezing responses during the CS and pre-CS period were evaluated statistically with two-factor ANOVAs with group (conditioned, control) as the between-subjects factor and experimental session as within-subject factor. A significant group–session interaction was observed for CS freezing ($F(6, 60) = 5.23$, $P < 0.001$) but not for pre-CS freezing ($F(6, 60) = 0.42$, $P > 0.1$) (not shown). Significant difference of post hoc analysis are indicated (*Duncan, $P < 0.05$, between groups). Freezing in sessions in which US presentations occur (sessions 3–5) is not useful as a measure of fear conditioning. Associative conditioning of freezing is best shown in session 6, in which only the CS was presented. The reduction in freezing in session 7 relative to session 6 reflects extinction of the CS–US association. The small amount of freezing exhibited by the control group after training (~5 s) reflects normal acquisition of freezing to the experimental context that extends into, but which is not elicited by, the CS^{8,10}. Error bars, $\pm$ s.e.m.

training ($F(6, 60) = 0.42$, $P > 0.1$). Conditioned animals showed more freezing during the CS than during the pre-CS period ($F(6, 60) = 5.2$, $P > 0.01$), whereas freezing did not differ during the pre-CS and CS periods in the control group ($F(6, 60) = 0.67$, $P > 0.1$). The elevated freezing to the CS relative to the pre-CS period in the conditioned group and the equivalence of freezing in the CS and pre-CS periods for the control group leads to two conclusions. First, freezing during the CS in the control group after training is caused by the experimental context and continues into but is not evoked by the CS. Second, freezing during the CS in the conditioned group is, at least in part, specifically related to the occurrence of the CS and its association with the US.

In previous studies of freely behaving rats, changes in hippocampal field potentials measured in the course of learning have been shown to be attributable in part to modulation of brain temperature by task-related changes in locomotor activity^{11,16}. Also, hippocampal field potentials are generally susceptible to modulation by behavioural state at the time of evoked potential sampling^{11,17–19}. The dramatic acquisition of freezing behaviour in the course of fear conditioning therefore raises the question of whether this learning-induced change in behaviour may produce the observed changes in CS-EPs in the LA through tonic effects related to brain temperature or other behaviourally related factors that merely coincide with field-potential measurements.

Despite a greater than 10-fold increase in freezing behaviour by both groups in the course of training compared with pre-training testing, and the corresponding increase in the proportion of freezing-coincident sampling of evoked potentials during the CS (from approximately 1 of 20 freezing-coincident samples for both groups in sessions 1 and 2, to approximately 12 of 20 freezing-coincident samples for both groups in session 5), only conditioned animals showed increases in CS-EPs during training with respect to baseline levels, and only in sessions with paired training (sessions 4 and 5); control group CS-EPs showed no significant change during any session, relative to baseline testing.

These data indicate that our measures of CS-EPs are not modulated by freezing expressed at the time of field-potential sampling, or

by possible behavioural modulation of brain temperature during the CS. The increases in slope and amplitude of CS-EPs measured in this experiment do not simply correlate with freezing behaviour, rather, they correlate with the presence of contingency information that identifies the CS as a danger signal, and with the degree to which the conditioned group makes use of this information after training.

As in our previous study of LTP and auditory evoked field potentials in the LA, the latency of CS-EPs measured in the present study varied between rats, but always fell within the latency range (15–30 ms) that invariably corresponded to histologically confirmed electrode placement within the LA⁷. The mean latency of CS-EPs across all rats (18.52 ± 3.58 ms) matched that measured in the LTP study (18.50 ± 2.65 ms), which used similar auditory stimulation parameters⁷, and these latencies were not altered by either LTP induction or fear conditioning. This indicates that the potentials recorded in the two studies reflect similar stimulus-locked responses from the same general population of cells. As noted above, anatomical and physiological evidence identified these field potentials as being locally generated in the LA. Further, the coincidence of the latency of the peak negativity of the evoked potentials with the latency of single neuron activity concurrently elicited by the auditory stimulus suggested that the negative-going component of the potentials reflects extracellular currents arising from local postsynaptic activation⁷. LTP induction in anaesthetized rats also produced effects of similar magnitude on both auditory evoked field potentials (change in slope over baseline, $+129.59 \pm 6.88\%$) and on electrical single-pulse stimulation (the typical test stimulus for LTP studies; change in slope over baseline, $+108.2 \pm 10.93\%$)⁷. Fear conditioning produced effects of similar magnitude on CS-EPs (change in slope over baseline, $+98.5 \pm 36.94\%$). Fear conditioning also alters single unit responses in the LA²⁰, and the conditioned changes in unit activity occur at latencies consistent with the changes we found in CS-EPs.

Our data indicate that CS-EPs in the LA reflect processes relevant to conditioned fear. In particular, to the extent that the negative-going slope can be interpreted as a measure of synaptic activation, we can conclude that fear conditioning, like LTP induction in CS pathways, potentiates synaptic currents. Because the same treatment potentiated both synaptic currents and conditioned fear behaviour over the same general time course, it is plausible that the enhancement of the field potentials reflects synaptic mechanisms that are responsible for the conditioning of fear behaviour. Processes mechanistically similar to LTP may therefore underlie the learning process which results from temporal association of the CS with the US, through which the CS comes to elicit conditioned fear responses.

Several previous studies have attempted to show that natural learning induces LTP-like changes in the hippocampus. In some of these studies, learning altered hippocampal physiology, but because the hippocampus is not required for the learned behaviour, the changes cannot account for learning^{21,22}. Other studies have used behavioural tasks that are dependent on the hippocampus²³, but interpretation of these data is limited by the poor understanding of the flow of task-relevant information in specific synaptic circuits within the hippocampus and the contribution of these circuits to the behaviour under study^{5,6}. In contrast, the well-defined and easily controlled sensory components of fear conditioning, and their tight coupling to mechanisms controlling the expression of learned fear responses, make this system well suited for such an analysis. We previously induced LTP in circuits known to be involved in fear conditioning⁷ and have now shown that fear conditioning alters neural activity in these circuits in the same way as LTP induction. Furthermore, we measured both artificial LTP and fear conditioning using an auditory test stimulus, which in the fear-conditioning experiment was the environmental cue that the animals learned to fear.

Other similarities exist between fear conditioning and the classic form of hippocampal LTP, which depends on glutamatergic

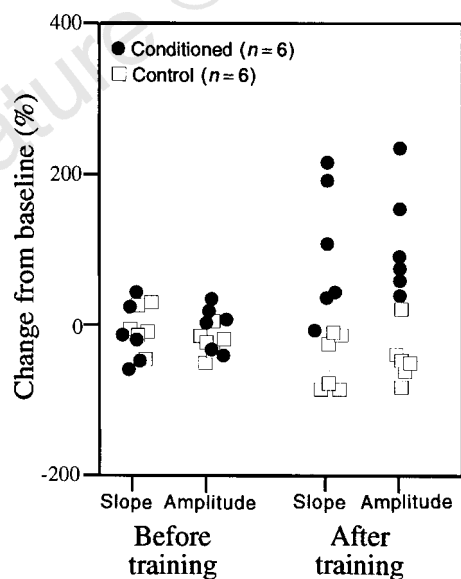


Figure 2 Scattergram of slope and amplitude values for each of the control and conditioned animals, before and after training. Pictured are normalized slope and amplitude values for each animal at the beginning of the experiment (testing, session 1), and in the first testing session after training (session 6). Points have been offset horizontally as needed to allow every point to be seen. Training had a reliable effect on the slope and amplitude of the CS-EP in the conditioned group, but not the control group.

mechanisms, particularly processes mediated by the NMDA (*N*-methyl-D-aspartate) receptor^{1,2}. CS processing in LA involves glutamatergic transmission^{24–26}, and the blockade of NMDA receptors in LA and adjacent regions interferes with fear conditioning^{27–29}. Also, facilitation of AMPA/NMDA receptor function modulates fear conditioning and hippocampal LTP in much the same way: both fear conditioning and LTP induction occur at an accelerated rate, but with no change in the final level of acquired conditioned fear or ceiling of potentiation⁹. Thus the LTP-like mechanisms engaged by fear conditioning may share mechanistic features with the more thoroughly studied, NMDA-dependent mechanisms known to be involved in hippocampal LTP, but which have been difficult to relate to hippocampal-dependent learning processes. It remains to be determined whether changes in synaptic strength produced in the amygdala by LTP induction and those produced by fear conditioning are both NMDA dependent. Such a demonstration would help to provide a mechanistic link between LTP and at least one form of memory. □

Methods

Surgery. Rats were anaesthetized and implanted with a stainless-steel recording electrode (0.6 mΩ) in the LA, and a ground electrode in the skull, under aseptic surgical conditions. The electrodes were mounted to the skull using dental cement. The wound was sutured and analgesics administered, and animals recovered for at least 5 days before the experiment.

Apparatus. The conditioning chamber was constructed of stainless-steel bars, acoustically transparent to the CS frequency. The chamber was kept within a ventilated and temperature-regulated acoustic isolation box lined with anechoic panels. Stimulus delivery and data acquisition were controlled by a custom-made Matlab application, using a Cambridge Electronics Devices 1401+. The isolation box was equipped with a video camera and VCR for recording of behaviour.

Conditioning protocol. The CS frequency was chosen so that the rat's head would be acoustically transparent to the CS, reducing the effect of head position on CS intensity at the tympani. The US (0.3 mA, 500 ms) was delivered through the floor of the conditioning chamber. In paired sessions, the US occurred immediately after the end of each CS. In unpaired sessions, the US occurred during the inter-CS interval (5 US per session; mean interval between CS and US, 78 s; range, 60–120 s). The sequence of testing and training sessions over 6 days is shown in Fig. 1.

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- Malenka, R. C. & Nicoll, R. A. NMDA-receptor-dependent synaptic plasticity: multiple forms and mechanisms. *Trends Neurosci.* **16**, 521–527 (1993).
- Bliss, T. V. P. & Collingridge, G. L. A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* **361**, 31–39 (1993).
- Brown, T. H. & Chattarji, S. in *Models of Neural Networks II* (eds Domany, E., Van Hemmen, J. L. & Schulten, K.) 287–314 (Springer-Verlag, New York, 1994).
- Stäubli, U. V. in *Brain and Memory: Modulation and Mediation of Neuroplasticity* (eds McGaugh, J. L., Weinberger, N. M. & Lynch, G.) 303–318 (Oxford Univ. Press, New York, 1995).
- Barnes, C. A. Involvement of LTP in memory: Are we "searching under the streetlight"? *Neuron* **15**, 751–754 (1995).
- Eichenbaum, H. The LTP–memory connection. *Nature* **378**, 131–132 (1995).
- Rogan, M. T. & LeDoux, J. E. LTP is accompanied by commensurate enhancement of auditory-evoked responses in a fear conditioning circuit. *Neuron* **15**, 127–136 (1995).
- Phillips, R. G. & LeDoux, J. E. Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. *Behav. Neurosci.* **106**, 274–285 (1992).
- Rogan, M. T., Stäubli, U. V. & LeDoux, J. E. AMPA-receptor facilitation accelerates fear learning without altering the level of conditioned fear acquired. *J. Neurosci.* **17**, 5928–5935 (1997).
- Kim, J. J. & Fanselow, M. S. Modality-specific retrograde amnesia of fear. *Science* **256**, 675–677 (1992).
- Moser, E. I., Moser, M.-B. & Andersen, P. Potentiation of dentate synapses initiated by exploratory learning in rats: dissociation from brain temperature, motor activity, and arousal. *Learn. Memory* **1**, 55–73 (1994).
- Blanchard, R. J. & Blanchard, D. C. Passive and active reactions to fear-eliciting stimuli. *J. Comp. Physiol. Psychol.* **68**, 129–135 (1969).
- Blanchard, R. J. & Blanchard, D. C. Crouching as an index of fear. *J. Comp. Physiol. Psychol.* **67**, 370–375 (1969).
- Bouton, M. E. & Bolles, R. C. Conditioned fear assessed by freezing and by the suppression of three different baselines. *Anim. Learn. Behav.* **8**, 429–434 (1980).
- Bolles, R. C. & Fanselow, M. S. A perceptual-defensive-recuperative model of fear and pain. *Behav. Brain Sci.* **3**, 291–323 (1980).
- Moser, E., Mathiesen, I. & Andersen, P. Association between brain temperature and dentate field potentials in exploring and swimming rats. *Science* **259**, 1324–1326 (1993).
- Winson, J. & Absug, C. Neuronal transmission through hippocampal pathways dependent on behavior. *J. Neurophysiol.* **41**, 716–732 (1978).
- Leung, S. Behavior-dependent evoked potentials in the hippocampal CA1 region of the rat. I. Correlation with behavior and EEG. *Brain Res.* **198**, 95–117 (1980).
- Buzsáki, G., Grastyán, E., Czopf, J., Kelenyi, L. & Prohaska, O. Changes in neuronal transmission in the rat hippocampus during behavior. *Brain Res.* **225**, 235–247 (1981).

- Quirk, G. J., Repp, J. C. & LeDoux, J. E. Fear conditioning enhances short-latency auditory responses of lateral amygdala neurons: parallel recordings in the freely behaving rat. *Neuron* **15**, 1029–1039 (1995).
- Skelton, R. W., Scarth, A. S., Wilkie, D. M., Miller, J. J. & Phillips, G. Long-term increases in dentate granule cell responsivity accompany operant conditioning. *J. Neurosci.* **7**, 3081–3087 (1987).
- Deadwyler, S. A., West, M. O., Christian, E., Hampson, R. E. & Foster, T. C. Sequence-related changes in sensory-evoked potentials in the dentate gyrus: as mechanism for item-specific short-term information storage in the hippocampus. *Behav. Neural Biol.* **44**, 201–212 (1985).
- Jeffrey, K. J. LTP and spatial learning—where to next? *Hippocampus* **7**, 95–110 (1997).
- Farb, C. R. & LeDoux, J. E. NMDA and AMPA receptors in the lateral nucleus of the amygdala are postsynaptic to auditory thalamic afferents. *Synapse* **27**, 106–121 (1997).
- Li, X., Phillips, R. G. & LeDoux, J. E. NMDA and non-NMDA receptors contribute to synaptic transmission between the medial geniculate body and the lateral nucleus of the amygdala. *Exp. Brain Res.* **105**, 87–100 (1995).
- Li, X. F., Stutzmann, G. E. & LeDoux, J. L. Convergent but temporally separated inputs to lateral amygdala neurons from the auditory thalamus and auditory cortex use different postsynaptic receptors: *in vivo* intracellular and extracellular recordings in fear conditioning pathways. *Learn. Memory* **3**, 229–242 (1996).
- Miserendino, M. J. D., Sananes, C. B., Melia, K. R. & Davis, M. Blocking of acquisition but not expression of conditioned fear-potentiated startle by NMDA antagonists in the amygdala. *Nature* **345**, 716–718 (1990).
- Maren, S., Aharonov, G., Stote, D. L. & Fanselow, M. S. *N*-Methyl-D-Aspartate receptors in the basolateral amygdala are required for both acquisition and expression of the conditional fear in rats. *Behav. Neurosci.* **110**, 1365–1374 (1996).
- Gewirtz, J. C. & Davis, M. Second-order fear conditioning prevented by blocking NMDA receptors in amygdala. *Nature* **388**, 471–473 (1997).
- Rogan, M. T. & LeDoux, J. E. Intra-amygdala infusion of APV blocks both auditory evoked potentials in the lateral amygdala and thalamo-amygdala transmission, but spares cortico-amygdala transmission. *Soc. Neurosci. Abstr.* **21**, 1930 (1995).

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Fear conditioning induces a lasting potentiation of synaptic currents *in vitro*

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The amygdala plays a critical role in the mediation of emotional responses, particularly fear, in both humans and animals^{1–4}. Fear conditioning, a conditioned learning paradigm, has served as a model for emotional learning in animals, and the neuroanatomical circuitry underlying the auditory fear-conditioning paradigm is well characterized⁵. Synaptic transmission in the medial geniculate nucleus (MGN) to lateral nucleus of the amygdala (LA) pathway, a key segment of the auditory fear conditioning circuit, is mediated largely through *N*-methyl-D-aspartate (NMDA) and non-NMDA (such as α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)) glutamate receptors⁶; the potential for neural plasticity in this pathway is suggested by its capacity to support long-term potentiation (LTP)^{7,8}. Here we report a long-lasting increase in the synaptic efficacy of the MGN–LA pathway attributable to fear-conditioning itself, rather than an electrically induced model of learning. Fear-conditioned animals show a presynaptic facilitation of AMPA-receptor-mediated transmission, directly measured *in vitro* with whole-cell recordings in lateral amygdala neurons. These findings represent one of the first *in vitro* measures of synaptic plasticity resulting from emotional learning by whole animals.

Fear-conditioned rats, when exposed to a tone (conditioned stimulus, CS) repeatedly paired with an aversive footshock (unconditioned stimulus, US), respond with a potentiated acoustic startle reflex ($+58.9\% \pm 11.6\%$, $n = 27$; see Methods) immediately following CS presentation, whereas unpaired control rats, exposed to the CS and US in an unpaired, pseudorandom fashion, do not ($+2.6\% \pm 5.6\%$, $n = 23$; unpaired *t*-test: $P < 0.0001$) (Fig. 1a). *In vivo* experiments suggest that the amygdala is involved in both the acquisition and expression of fear-potentiated startle^{9–11}. We prepared coronal slices from fear-conditioned rats 24 hours after

completion of behaviour testing, as well as from experimentally naive and unpaired control rats, to investigate the synaptic alterations induced by whole-animal emotional learning.

Excitatory postsynaptic currents (EPSCs) in lateral amygdala neurons were elicited by stimulating fibres emerging from the internal capsule, shown to carry efferents from the medial geniculate nucleus¹². The EPSC was completely blocked by co-application of 50 μ M D-2-amino-5-phosphovaleate (D-AP5; an NMDA-receptor antagonist) and 100 μ M GYKI 52466 (a non-competitive AMPA-receptor antagonist¹³; $n = 3$), suggesting mediation by both NMDA and AMPA receptors. The EPSC threshold was significantly lower in fear-conditioned (FC) animals (4.6 ± 0.2 V, $n = 23$) than in naive controls (NC: 5.5 ± 0.2 V, $P < 0.01$, $n = 28$) or in unpaired controls (UC: 5.4 ± 0.2 V, $P < 0.01$, $n = 24$) animals. Action potential threshold (FC: 9.0 ± 0.4 V, NC: 9.5 ± 0.4 V, UC: 10.2 ± 0.5 V) and

input resistance (FC: 138.9 ± 7.3 M Ω , NC: 136.5 ± 8.5 M Ω , UC: 133.1 ± 10.7 M Ω) did not differ between the three groups.

EPSCs are potentiated in neurons from fear-conditioned rats relative to EPSCs recorded in neurons from naive and unpaired controls (Fig. 1b). This potentiation is reflected both in the composite response, a measure of the native synaptic response, and in the AMPA-receptor-mediated component of the EPSC (Fig. 1c). Input-output relationships, measuring EPSC amplitude (pA, output) as a function of afferent fibre stimulus intensity (V, input) for each neuron, were compared in the three groups (naive control, unpaired control and fear conditioned) using a one-way ANOVA. The effect of treatment (fear-conditioned, unpaired control, or naive control) was significant ($F(2, 51) = 26.27$, $P < 0.0001$). Whereas the slopes of the curves from naive controls (15.1 ± 1.2 pA/V, $n = 14$) and unpaired controls (11.2 ± 1.0 pA/V,

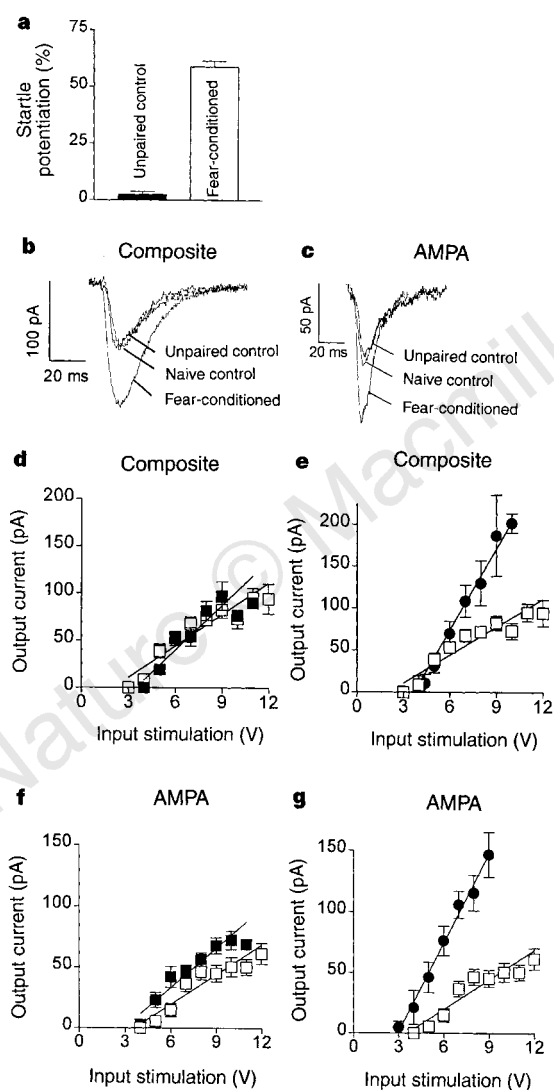


Figure 1 Fear conditioning results in potentiation of evoked EPSCs. **a**, Plot of per cent startle potentiation in fear-conditioned and unpaired control animals. **b**, Composite EPSCs evoked with input stimulations of 9 V. **c**, AMPA EPSCs evoked with input stimulations of 9 V. **d**, Input-output curves for composite EPSCs in naive control ($\blacksquare$, $n = 14$) and unpaired control ($\square$, $n = 23$) animals. **e**, Input-output curves for composite EPSCs in unpaired control ($\square$, $n = 23$) and fear conditioned ($\bullet$, $n = 22$) animals. **f**, Input-output curves for AMPA EPSCs in unpaired control ($\square$, $n = 16$) and naive control ($\blacksquare$, $n = 12$) animals. **g**, Input-output curves for AMPA EPSCs in unpaired control ($\square$, $n = 16$) and fear conditioned ($\blacksquare$, $n = 17$) animals.

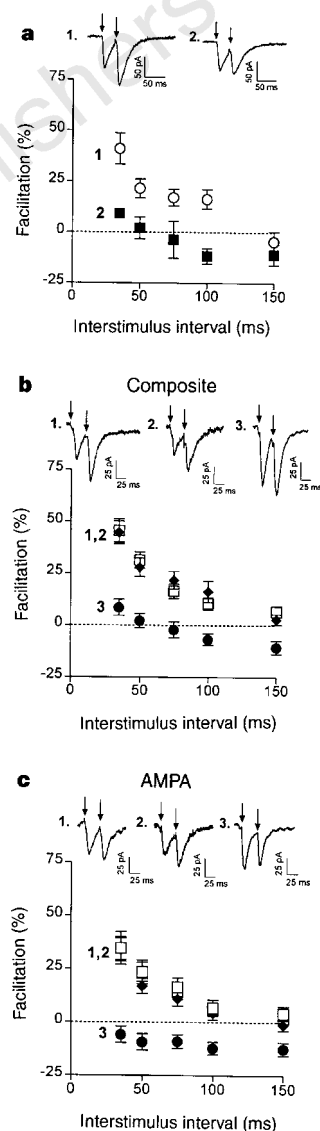


Figure 2 PPF is decreased in fear-conditioned animals. Numbered traces in **a**, **b** and **c** correspond to numbers in the plot; traces shown are averages of five recorded traces. $V_{\text{HOLD}} = -60$ mV. **a**, Decreasing the external $[\text{Mg}^{2+}]/[\text{Ca}^{2+}]$ ratio from 0.48 ($\square$) to 0.16 ($\blacksquare$) decreases the amount of PPF in naive control animals ($n = 4$). **b**, PPF is significantly decreased in fear-conditioned animals ($\bullet$, $n = 18$) compared to naive ($\blacklozenge$, $n = 16$) and unpaired ($\square$, $n = 22$) controls. **c**, PPF of the AMPA component is significantly decreased in fear-conditioned animals ($\bullet$, $n = 17$) compared to naive ($\blacklozenge$, $n = 11$) and unpaired ($\square$, $n = 17$) controls.

$n = 23$) were not significantly different ($P > 0.05$, Fig. 1d), the slope of the input–output curve in neurons from fear-conditioned animals (32.3 ± 3.7 pA/V, $n = 22$) was significantly greater than that in neurons from unpaired control animals ($P < 0.001$; Newman–Keuls post test). These data suggest an increase in synaptic efficacy in lateral amygdala neurons from fear-conditioned animals (Fig. 1e).

As many reports on LTP, a model of learning, suggest a predominant facilitation of AMPA-receptor-mediated transmission^{14–16}, and as AMPA receptors have been proposed to mediate the expression of fear conditioning *in vivo*¹⁰, we focused on the

AMPA component of the EPSC by recording input–output curves in the presence of D-AP5 (50 μ M). Comparing the slopes of the input–output curves in the three groups with a one-way ANOVA showed that the effect of treatment was significant ($F(2, 35) = 18.5$, $P < 0.0001$). Whereas the slope of the AMPA component curve in unpaired control animals (8.2 ± 0.9 pA/V, $n = 16$) did not differ ($P > 0.05$) from that in the naive control animals (10.9 ± 0.9 pA/V, $n = 12$) (Fig. 1f), the slope of the input–output curve in fear-conditioned animals (24.3 ± 2.7 pA/V, $n = 17$) was significantly ($P < 0.001$; Newman–Keuls post test) greater than that of the curve from unpaired control animals, suggesting that AMPA-mediated transmission in the amygdala is potentiated in emotional learning (Fig. 1g).

Fear conditioning results in a potentiation of the evoked MGN–LA response. To examine whether increases in presynaptic neurotransmitter release could be a contributing factor in this potentiation, we analysed paired-pulse facilitation (PPF) in neurons from the three groups. PPF is a phenomenon by which a second synaptic stimulation of equal magnitude evokes a larger synaptic response than the first, if the interval between the two pulses is sufficiently brief. PPF has traditionally been attributed to short-term increases in presynaptic intracellular calcium levels, and has been used as a tool to implicate presynaptic involvement; manipulations that increase the probability (p) of transmitter release, such as decreasing the external $[Mg^{2+}]/[Ca^{2+}]$ ratio, have been shown to decrease PPF^{17–21}. Decreasing the external $[Mg^{2+}]/[Ca^{2+}]$ ratio from 0.48 to 0.16 produced the expected decrease in the degree of PPF at this MGN–LA synapse ($n = 4$) (Fig. 2a). Comparison of the time course and magnitude of PPF between the three experimental groups showed that the effect of treatment group was significant ($F(2, 225) = 37.26$, $P < 0.0001$, two-way ANOVA). A significant treatment effect ($F(2, 55) = 13.89$, $P < 0.01$, one-way ANOVA) was observed at the interstimulus interval showing maximal PPF, 35 ms, with neurons from fear-conditioned animals ($n = 18$) showing significantly less PPF than neurons from naive ($P < 0.001$, $n = 16$; Newman–Keuls) and unpaired ($P < 0.001$, $n = 22$; Newman–Keuls) control animals. PPF in neurons from naive and unpaired control animals did not differ significantly ($P > 0.05$) (Fig. 2b).

Some studies report a postsynaptic contribution to PPF mediated through NMDA receptors, although PPF mediated through AMPA receptors is thought to be primarily presynaptic^{22–24}. Presynaptic regulation of AMPA PPF at this synapse was confirmed by the lack of correlation between EPSC1 and EPSC2 amplitudes and a lack of postsynaptic voltage dependence²⁵. To assess AMPA-mediated PPF, we analysed PPF in neurons from the three groups of animals in the presence of D-AP5 (50 μ M). Comparison of the time course and magnitude of PPF between groups indicated that the effect of treatment group was significant ($F(2, 210) = 76.50$, $P < 0.0001$, two-way ANOVA). The effect of treatment was significant ($F(2, 41) = 22.48$, $P < 0.0001$, one-way ANOVA) at the 35-ms interstimulus interval, with neurons from fear-conditioned animals ($n = 17$) exhibiting significantly less PPF of the AMPA EPSC than those from naive ($P < 0.001$, $n = 11$; Newman–Keuls) and unpaired ($P < 0.001$, $n = 17$; Newman–Keuls) control animals. PPF in neurons from naive and unpaired controls did not differ significantly ($P > 0.05$) (Fig. 2c). These data, coupled with the quantitative and qualitative similarity of PPF in fear-conditioned animals and PPF in decreased external $[Mg^{2+}]/[Ca^{2+}]$, suggest that increased presynaptic release contributes to synaptic potentiation in lateral amygdala neurons from fear conditioned animals.

The lack of synaptic potentiation in the unpaired control animals suggests that potentiation observed in fear-conditioned animals is not the result of nonspecific exposure to stress. However, it does not rule out the possibility that potentiation is a nonspecific result of the learning paradigm, not limited to fear-conditioning circuitry. To test this, we recorded EPSCs evoked in lateral amygdala neurons by

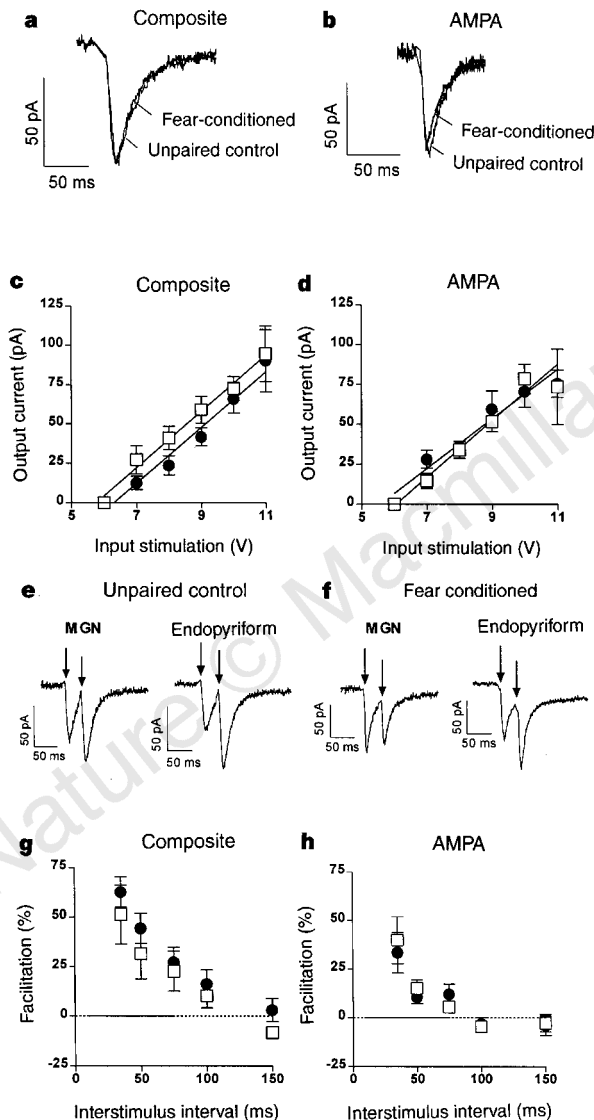


Figure 3 The endopyriform–LA pathway is not potentiated in fear-conditioned animals. **a**, Example composite EPSCs evoked with an input stimulation of 10 V. **b**, Example AMPA EPSCs evoked with an input stimulation of 10 V. **c**, Input–output curves for composite EPSCs in unpaired control ($\square$, $n = 6$) and fear-conditioned ($\bullet$, $n = 6$) animals. **d**, Input–output curves for the AMPA EPSC in unpaired control ($\square$, $n = 5$) and fear-conditioned ($\bullet$, $n = 6$) animals. **e**, The same neuron from an unpaired control animal displays PPF in both the MGN–LA pathway and the endopyriform–LA pathway. **f**, Fear-conditioned animals display a selective loss of PPF in the MGN–LA pathway while maintaining PPF in the endopyriform–LA pathway. **g**, PPF does not differ in fear-conditioned animals ($\bullet$, $n = 5$) and unpaired controls ($\square$, $n = 6$). **h**, PPF of the AMPA component does not differ in fear-conditioned animals ($\bullet$, $n = 5$) and unpaired controls ($\square$, $n = 5$).

stimulating the endopyriform nucleus, a structure not believed to be involved in fear-conditioning. In this pathway, neither the composite nor the AMPA EPSC exhibited potentiation in fear-conditioned animals relative to unpaired controls (Fig. 3a, b). The slope of the input–output curves did not differ between fear-conditioned and unpaired control animals for the composite EPSC ($F(1, 61) = 0.006$, $P = 0.940$, one-way ANOVA) or the AMPA EPSC ($F(1, 50) = 0.616$, $P = 0.563$, one-way ANOVA) (Fig. 3c, d).

The endopyriform–lateral amygdala pathway also displayed PPF. In the same neuron, PPF was evident in both the MGN–LA pathway and the endopyriform–LA pathway in unpaired control animals; a paired t -test revealed that the degree of facilitation at the 35-ms interstimulus interval did not differ between the two inputs (MGN: $+55.3\% \pm 20.6\%$, endopyriform: $+56.5\% \pm 30.0\%$, $n = 3$, $P = 0.98$) (Fig. 3e). However, neurons from fear-conditioned animals show a selective loss of PPF in the MGN–LA pathway (MGN: $-0.3 \pm 4.8\%$, endopyriform: $+62.7 \pm 7.6\%$, $n = 5$, $P < 0.001$, paired t -test) (Fig. 3f). The overall time course and magnitude of PPF in the endopyriform–LA pathway did not differ between neurons from unpaired control animals and fear-conditioned animals either for the composite EPSC ($F(1, 45) = 2.49$, $P = 0.121$) or the AMPA EPSC ($F(1, 35) = 0.82$, $P = 0.817$, two-way ANOVA) (Fig. 3g, h). The finding that potentiation in fear-conditioned animals is specific to synapses involved in fear-conditioning circuitry provides additional support for this potentiation serving as a substrate for emotional learning.

The findings presented here provide direct evidence that the learning associated with fear-conditioning produces changes in the MGN–LA synapse that can be measured *in vitro*. The synaptic potentiation associated with fear-conditioning is the result, at least in part, of a presynaptic increase in transmitter release; potential postsynaptic changes occurring with fear conditioning remain to be investigated. Furthermore, the lack of potentiation in the endopyriform–LA pathway in fear-conditioned animals provides strong evidence for the specificity of learning-induced synaptic potentiation to fear-conditioning circuitry. The ability to record and analyse the expression of whole-animal emotional learning in an *in vitro* slice, using many of the same parameters traditionally used in the study of learning models such as LTP, represents an important means of revealing the neural basis of behaviour. □

Methods

Slice preparation. *In vitro* brain slices were obtained from male Sprague–Dawley rats (weight range, 100–150 g) and prepared as described²⁶. Rats were decapitated and the brains were removed and cooled rapidly in a modified ACSF solution (0–6°C) bubbled continuously with 95% O₂ and 5% CO₂. Three to four serial coronal slices of 500 µm thickness per hemisphere were cut with a Vibroslice. In the recording chamber, the slice was fully submerged and continuously perfused with oxygenated ACSF maintained at $32 \pm 2^\circ\text{C}$. Modified ACSF is (millimolar): NaCl, 117; KCl, 4.7; CaCl₂, 2.5; MgCl₂, 1.2; NaHCO₃, 25; NaH₂PO₄, 1.2; and glucose, 11.5.

Whole-cell recording. Voltage-clamp recordings in the whole-cell configuration were obtained as described²⁷. Neurons were recorded in discontinuous single electrode whole-cell voltage clamp. The composition of the internal electrode solution was: Cs-gluconate, 115 mM; NaCl, 5 mM; EGTA, 1 mM; CaCl₂, 0.3 mM; MgCl₂, 2 mM; Na-ATP, 5 mM; Na-GTP, 0.4 mM; HEPES 10 mM; the internal solution was adjusted to a pH of 7.2 and osmolality of 280 mOsm; tip resistance was 4–5 MΩ.

Stimulation and recording parameters. Bipolar concentric stimulating electrodes were placed (1) on fibres emerging from the internal capsule which originate in the medial geniculate nucleus of the thalamus and project monosynaptically to the lateral nucleus of the amygdala, and (2) within the endopyriform nucleus. Neurons used for recording were located in the dorsal portion of the lateral amygdala, where fibres from the medial geniculate terminate. Input–output curves were constructed by varying stimulus intensity and measuring EPSC amplitude from EPSC threshold to spike threshold. Peak EPSC amplitude was measured as the peak inward current within a time

window defined as current onset to return to baseline. Peak amplitude for individual responses in the paired-pulse paradigm was measured as the difference between the current level before the stimulus artefact and the peak of the EPSC²⁸. Paired-pulse facilitation (PPF) was calculated as $[(\text{EPSC}_2 - \text{EPSC}_1)/\text{EPSC}_1] \times 100$. Paired-pulse EPSCs were elicited at a frequency of 0.1 Hz.

Fear conditioning. Fear conditioning was measured using the potentiated startle paradigm²⁹. Male Sprague–Dawley rats (100–150 g) were trained and tested in a stabilimeter device, in which cage movement results in the displacement of an accelerometer located beneath the stabilimeter (San Diego Instruments). Startle amplitude was defined as peak accelerometer voltage within 200 ms after startle stimulus onset. 50-ms bursts of white noise at 95 dB were used as the acoustic startle stimulus. The conditioned stimulus (CS) was a 3.7 s 70-dB white-noise bandpass filtered with low and high passes at 2,000 Hz (24 dB per octave attenuation). This stimulus was paired 10 times a day for two days with a 0.5-mA footshock of 0.5 s in experimental animals to provide the aversive component necessary for fear conditioning. Unpaired controls received the same number of CS and US presentations, but in an unpaired, pseudorandom fashion. Amygdala slices were prepared from animals 24 h after the final testing session; 1–2 neurons were recorded per animal, typically with a single neuron recorded per slice. To investigate possible experimenter bias, experiments on 5 fear-conditioned and 5 unpaired animals were blinded to animal group during data acquisition and analysis. The data from these experiments were not significantly different from those obtained during non-blinded experiments, so all data were pooled.

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1. Scott, S. K. *et al.* Impaired auditory recognition of fear and anger following bilateral amygdala lesions. *Nature* **385**, 254–257 (1997).
2. Aldolphs, R., Tranel, D., Damasio, H. & Damasio, A. Impaired recognition of emotion in facial expressions following bilateral damage to the human amygdala. *Nature* **372**, 669–672 (1994).
3. Maren, S. Synaptic transmission and plasticity in the amygdala: An emerging physiology of fear conditioning circuits. *Mol. Neurobiol.* **13**, 1–22 (1996).
4. Davis, M., Rainnie, D. & Cassell, M. Neurotransmission in the rat amygdala related to fear and anxiety. *Trends Neurosci.* **17**, 208–214 (1994).
5. LeDoux, J. E. Emotion: clues from the brain. *Annu. Rev. Psychol.* **46**, 209–235 (1995).
6. Li, X. F., Phillips, R. & LeDoux, J. E. NMDA and non-NMDA receptors contribute to synaptic transmission between the medial geniculate body and the lateral nucleus of the amygdala. *Exp. Brain Res.* **105**, 87–100 (1995).
7. Clugnet, M. C. & LeDoux, J. E. Synaptic plasticity in fear conditioning circuits: induction of LTP in the lateral nucleus of the amygdala by stimulation of the medial geniculate body. *J. Neurosci.* **10**, 2818–2824 (1990).
8. Rogan, M. T. & LeDoux, J. E. LTP is accompanied by commensurate enhancement of auditory-evoked responses in a fear conditioning circuit. *Neuron* **15**, 127–136 (1995).
9. Davis, M., Falls, W. A., Campeau, S. & Munson, K. Fear-potentiated startle: a neural and pharmacological analysis. *Behav. Brain Res.* **58**, 175–198 (1993).
10. Kim, M., Campeau, S., Falls, W. A. & Davis, M. Infusion of the non-NMDA receptor antagonist CNQX into the amygdala blocks the expression of fear-potentiated startle. *Behav. Neural Biol.* **59**, 5–8 (1993).
11. Miserendino, M. J. D., Sananes, C. B., Melia, K. R. & Davis, M. Blocking of acquisition but not expression of conditioned fear-potentiated startle by NMDA antagonists in the amygdala. *Nature* **345**, 716–718 (1990).
12. LeDoux, J. E., Ruggiero, D. A. & Reis, D. J. Projections from anatomically defined regions of the medial geniculate body in the rat. *J. Comp. Neurol.* **242**, 182–213 (1985).
13. Parsons, C. G., Gruner, R. & Rozental, J. Comparative patch clamp studies on the kinetics and selectivity of glutamate receptor antagonism by 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzof[quinoxaline] (NBQX) and 1-(4-aminophenyl)-4-methyl-7,8-methyl-endoxyl-5H-2,3-benzodiazepine (GYKI 52466). *Neuropharmacology* **33**, 589–604 (1994).
14. Muller, D., Joly, M. & Lynch, G. Differential contributions of quisqualate versus NMDA receptors to the induction and expression of long-term potentiation. *Science* **242**, 1694–1697 (1988).
15. Kauer, J., Malenka, R. & Nicoll, R. A persistent postsynaptic modification mediates long-term potentiation in the hippocampus. *Neuron* **1**, 911–917 (1988).
16. Kullman, D. Amplitude fluctuations of dual-component EPSCs in hippocampal pyramidal cells: Implications for long-term potentiation. *Neuron* **12**, 1111–1120 (1994).
17. Katz, B. & Miledi, R. The role of calcium in neuromuscular facilitation. *J. Physiol.* **195**, 481–492 (1968).
18. Creager, R., Dunwiddie, T. & Lynch, G. Paired-pulse and frequency facilitation in the CA1 region of the *in vitro* rat hippocampus. *J. Physiol.* **299**, 409–424 (1980).
19. Manabe, T., Wyllie, D. J. A., Perkel, D. J. & Nicoll, R. A. Modulation of synaptic transmission and long-term potentiation: effects on paired-pulse facilitation and EPSC variance in the CA1 region of the hippocampus. *J. Neurophys.* **70**, 1451–1459 (1993).
20. Kuhnt, U. & Voronin, L. L. Interaction between paired-pulse facilitation and long-term potentiation in area CA1 of guinea-pig hippocampal slices: application of quantal analysis. *Neuroscience* **62**, 391–397 (1994).
21. Shulz, P., Cook, E. & Johnston, D. Changes in paired-pulse facilitation suggest presynaptic involvement in long-term potentiation. *J. Neurosci.* **14**, 5325–5337 (1994).
22. Clark, K. A., Randall, A. D. & Collinridge, G. L. A comparison of paired-pulse facilitation of AMPA and NMDA receptor-mediated excitatory postsynaptic currents in the hippocampus. *Exp. Brain Res.* **101**, 272–278 (1994).
23. Muller, D. & Lynch, G. Synaptic modulation of N-methyl-D-aspartate receptor-mediated responses in hippocampus. *Synapse* **5**, 94–103 (1990).
24. Lin, J. & Faber, D. Synaptic transmission mediated by single club endings on the goldfish Mauthner cell. II. Plasticity of excitatory postsynaptic potentials. *J. Neurosci.* **8**, 1313–1325 (1988).

25. McKernan, M. & Shinnick-Gallagher, P. Paired-pulse facilitation (PPF) in an internal capsule-lateral amygdala pathway. *Soc. Neurosci. Abstr.* **22**, 1536 (1996).
26. Neugebauer, V., Keele, N. B. & Shinnick-Gallagher, P. Epileptogenesis *in vivo* enhances the sensitivity of inhibitory presynaptic metabotropic glutamate receptors in basolateral amygdala neurons *in vitro*. *J. Neurosci.* **17**, 983–995 (1997).
27. Blanton, M. G., Lo Turco, J. J. & Kriegstein, A. R. Whole cell recording from neurons in slices of reptilian and mammalian cerebral cortex. *J. Neurosci. Meth.* **30**, 203–210 (1989).
28. Andreasen, M. & Häblitz, J. Paired-pulse facilitation in the dentate gyrus: A patch-clamp study in the rat hippocampus *in vitro*. *J. Neurophysiol.* **72**, 326–336 (1994).
29. Casella, J. V. & Davis, M. The design and calibration of a startle measurement system. *Physiol. and Behav.* **36**, 377–383 (1986).

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How opioids inhibit GABA-mediated neurotransmission

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The midbrain region periaqueductal grey (PAG) is rich in opioid receptors and endogenous opioids and is a major target of analgesic action in the central nervous system¹. It has been proposed that the analgesic effect of opioids on the PAG works by suppressing the inhibitory influence of the neurotransmitter GABA (γ -aminobutyric acid) on neurons that form part of a descending antinociceptive pathway². Opioids inhibit GABA-mediated (GABAergic) synaptic transmission in the PAG and other brain regions by reducing the probability of presynaptic neurotransmitter release^{3,4}, but the mechanisms involved remain uncertain. Here we report that opioid inhibition of GABAergic synaptic currents in the PAG is controlled by a presynaptic voltage-dependent potassium conductance. Opioid receptors of the μ type in GABAergic presynaptic terminals are specifically coupled to this potassium conductance by a pathway involving phospholipase A₂, arachidonic acid and 12-lipoxygenase. Furthermore, opioid inhibition of GABAergic synaptic transmission is potentiated by inhibitors of the enzymes cyclooxygenase and 5-lipoxygenase, presumably because more arachidonic acid is available for conversion to 12-lipoxygenase products. These mechanisms account for the analgesic action of cyclooxygenase inhibitors in the PAG⁵ and their synergism with opioids⁶.

To determine the presynaptic mechanism of opioid inhibition of GABAergic synaptic transmission, we examined the action of opioids on spontaneous action-potential-independent miniature GABAergic postsynaptic currents (mIPSCs) in the rat PAG. Superfusion of methionine-enkephalin (ME; 10 μ M) reduced the frequency of mIPSCs by $64 \pm 3\%$, without any significant reduction in their mean amplitude ($8 \pm 6\%$) in all PAG neurons tested (Fig. 1a–d; $n = 15$)⁴. This effect was mimicked by the μ agonist DAMGO ([D-Ala², N-Me-Phe⁴, Gly⁵-ol]-enkephalin; 0.1–1 μ M; Fig. 3b) and was abolished by the μ antagonist CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Pen-Thr-NH₂; 1 μ M; $n = 5$). The decrease in mIPSC frequency without any change in mIPSC amplitude is indicative of a reduction in the probability of transmitter release from presynaptic GABAergic terminals mediated by μ -receptors. Although opioid modulation of postsynaptic Ca²⁺ conductances and Ba²⁺-sensitive, inwardly rectifying K⁺ conductances is well established⁷, opioid inhibition of GABAergic synaptic transmission in PAG is not mediated by similar conductances in presynaptic terminals⁴. ME produced a significant reduction in mIPSC frequency in Ca²⁺-free, high-Mg²⁺ (10 mM)

solutions containing Cd²⁺ (100 μ M; $n = 4$), or Ba²⁺ (10 mM; $n = 4$) (Fig. 1e). Similar observations in cultured hippocampal neurons have led to the proposition that opioids directly inhibit the spontaneous GABA exocytotic process, without modulation of presynaptic Ca²⁺ or K⁺ conductances³. However, opioids can potentially modulate several K⁺ conductances⁸. We therefore examined the effects of other K⁺-channel blockers on presynaptic inhibition by opioids and found that the ME-induced reduction in mIPSC frequency was blocked by the voltage-dependent K⁺ channel blockers 4-aminopyridine (4-AP, 0.1–1 mM; $n = 9$) and dendrotoxin (100 nM; $n = 5$), but was unaffected by tetraethylammonium chloride (TEA; 10 mM; $n = 4$) (Fig. 1a–e)⁹.

We tested the specificity of the effect of these K⁺-channel blockers on inhibition of synaptic transmission in PAG. The GABA_B-receptor agonist baclofen (10 μ M) reduced the frequency of mIPSCs by $74 \pm 3\%$ ($n = 10$), without any significant reduction in their amplitude ($9 \pm 5\%$; Fig. 1a–e)¹⁰; baclofen also reduced mIPSC frequency in the presence of 4-AP (1 mM; $n = 5$) and dendrotoxin (100 nM; $n = 4$; Fig. 1a–e). Furthermore, ME (10 μ M) reduced the frequency of glutamatergic miniature excitatory postsynaptic currents (mEPSC) in the absence ($53 \pm 3\%$; $n = 6$)⁴ or presence of 4-AP (1 mM; $59 \pm 7\%$; $n = 6$). Thus, 4-AP and dendrotoxin selectively abolished presynaptic μ -receptor-mediated inhibition without affecting either GABA_B-receptor-mediated inhibition of GABA_A-ergic mIPSC frequency, or μ -receptor inhibition of glutamatergic mEPSC frequency.

Electrically evoked synaptic transmission is dependent on Ca²⁺ entry, so the mechanism of opioid inhibition of mIPSCs and electrically evoked GABAergic postsynaptic currents (eIPSCs) might differ. ME inhibited the amplitude of eIPSCs by $69 \pm 7\%$ ($n = 6$), and this was reduced by 4-AP (100 μ M; $16 \pm 5\%$ inhibition; $n = 6$) and dendrotoxin (100 nM; $26 \pm 12\%$ inhibition; $n = 5$; Fig. 1f–h). Baclofen reduced the amplitude of eIPSCs in the absence ($87 \pm 3\%$ inhibition; $n = 4$) or presence of 4-AP ($63 \pm 3\%$ inhibition; $n = 5$) and dendrotoxin ($85 \pm 3\%$ inhibition; $n = 4$; Fig. 1f–h). Thus, it is likely that the μ -opioid inhibition of electrically evoked GABA release and of spontaneous action potential independent GABA release in PAG results from a common mechanism.

These data define the site of the inhibitory action of opioids on GABAergic neurotransmission in PAG as a dendrotoxin- and 4-AP-sensitive, presumably Shaker-like⁹, voltage-dependent K⁺ conductance in presynaptic terminals. The mechanism by which a presynaptic K⁺ conductance regulates mIPSC frequency (in the absence of Ca²⁺ entry) remains to be determined, but probably involves hyperpolarization resulting from enhanced K⁺-channel activity. A similar mechanism of presynaptic inhibition might occur in other brain regions. Inhibition of electrically evoked excitatory postsynaptic currents in hippocampal CA3 neurons that is mediated by opioid receptor of the κ type is also abolished by voltage-dependent K⁺-channel blockers¹¹, although this could have been due to actions in somata or terminals. Inhibition of this presynaptic K⁺ conductance does not generalize to all forms of μ -opioid or other G-protein-receptor-mediated forms of presynaptic inhibition in PAG. μ -Opioid-mediated inhibition of glutamatergic transmission and GABA_B-receptor-mediated inhibition of GABAergic neurotransmission in PAG are mediated by as yet unidentified mechanisms because neither was abolished by 4-AP.

A variety of G-protein-modulated second-messenger pathways could be involved in the presynaptic opioid action. The sulphydryl alkylating agent N-ethylmaleimide (NEM) blocked the reduction in mIPSC frequency produced by ME ($n = 4$) and baclofen ($n = 3$) (Fig. 2f). However, NEM disrupts a number of cellular proteins in addition to pertussis-toxin-sensitive G-protein-mediated actions¹². Both the cyclic-AMP-dependent protein kinase A and protein kinase C systems regulate synaptic transmission¹³. We therefore examined whether maximal concentrations of activators of protein

kinases occluded presynaptic opioid action. Activators of protein kinase A (forskolin (10 μ M) and 8-bromo-cAMP (1 mM)) and of protein kinase C (phorbol 12,13-dibutyrate (PDBu; 0.3–1 μ M)) increased the frequency of mIPSCs by $386 \pm 68\%$ ($n = 5$), $195 \pm 31\%$ ($n = 4$) and $691 \pm 120\%$ ($n = 5$), respectively, without significantly affecting their amplitude ($1 \pm 5\%$ reduction; pooled $n = 14$; Fig. 2a). However, inhibition by ME and baclofen of mIPSC frequency in slices treated with forskolin, 8-bromo-cAMP or PDBu was comparable to that in unexposed PAG slices (Fig. 2a, f). Also, ME reduced mIPSC frequency in the presence of the protein kinase inhibitor staurosporin at 1 μ M ($n = 5$), a concentration that blocked forskolin- and PDBu-mediated increases in mIPSC frequency ($140 \pm 40\%$ of control; $n = 5$; Fig. 2a, f). These observations suggest that neither the μ -receptor- nor the GABA_B-receptor-mediated presynaptic inhibition results from modulation of protein kinase A or C activity, but instead are mediated by some other G-protein-coupled mechanism.

Opioid agonists liberate arachidonic acid in CHO cells transfected with μ receptors¹⁴, so we tested the effect of inhibitors of the phospholipase A₂ (PLA₂)/arachidonic acid pathway (Fig. 2g)^{15,16} on presynaptic opioid action. The PLA₂ inhibitors quinacrine (30 μ M; $n = 6$) and 4-bromo-phenacylbromide (4-BPB; 10 μ M; $n = 5$) abolished this action. While quinacrine and 4-BPB have other nonspecific effects^{16,17}, baclofen-induced presynaptic action was largely unaffected by the PLA₂ inhibitors ($n = 5$) (Fig. 2f). Thus, arachidonic acid or one of its metabolites probably mediates the opioid inhibition of mIPSC frequency.

Cyclooxygenase, lipoxygenase and epoxygenase pathways for arachidonic acid metabolism have been identified in the brain

(Fig. 2g)¹⁸. Superfusion of indomethacin (3–10 μ M; $n = 6$) or aspirin (30 μ M; $n = 5$) to block cyclooxygenase action did not significantly impair ME-induced reduction of mIPSC frequency (Fig. 2f). Superfusion of the lipoxygenase inhibitors nordihydroguaiaretic acid (NDGA; 10 μ M; $n = 4$) and eicosatrienoic acid (ETI; 6 μ M, $n = 4$) abolished the ME-induced reduction in mIPSC frequency (Fig. 2f). The 5-lipoxygenase inhibitors AA-861 (10 μ M, $n = 5$) and caffeic acid (10–30 μ M; $n = 5$), and L-655,238 (3 μ M; $n = 7$)¹⁹, an inhibitor of 5-lipoxygenase-activating protein, did not block this ME-induced reduction in mIPSC frequency (Fig. 2f), whereas the 12-lipoxygenase inhibitor baicalein (3–10 μ M) did ($n = 7$; Fig. 2b, f); baicalein also blocked the ME-induced reduction in eIPSC amplitude ($8 \pm 16\%$ inhibition; $n = 5$; Fig. 2c). Inhibition of the 12-lipoxygenase pathway selectively blocked opioid inhibition of GABAergic synaptic currents. Thus, baclofen-induced reduction in mIPSC frequency was unaffected by NDGA ($n = 3$) or baicalein ($n = 7$) (Fig. 2f), and ME-induced reduction in mIPSC frequency was unaffected by baicalein ($48 \pm 6\%$ inhibition; $n = 4$).

We next investigated whether arachidonic acid and its metabolites mimicked the presynaptic inhibitory effects of μ opioids. Superfusion of arachidonic acid (150 μ M) reduced the frequency of mIPSCs by $24 \pm 3\%$, without significantly reducing their mean amplitude ($4 \pm 2\%$; $n = 8$). This reduction in mIPSC frequency was blocked by baicalein (3–10 μ M; $-3 \pm 10\%$ inhibition; $n = 4$) (Fig. 2b) and was potentiated by indomethacin plus caffeic acid (10 μ M and 30 μ M, respectively; $42 \pm 5\%$ inhibition; $n = 4$) (Fig. 2d). In the presence of arachidonic acid, ME produced a non-additive inhibition of mIPSC frequency ($51 \pm 4\%$ inhibition; $n = 4$; Fig. 2d). Arachidonic acid is converted to hydroperoxyeico-

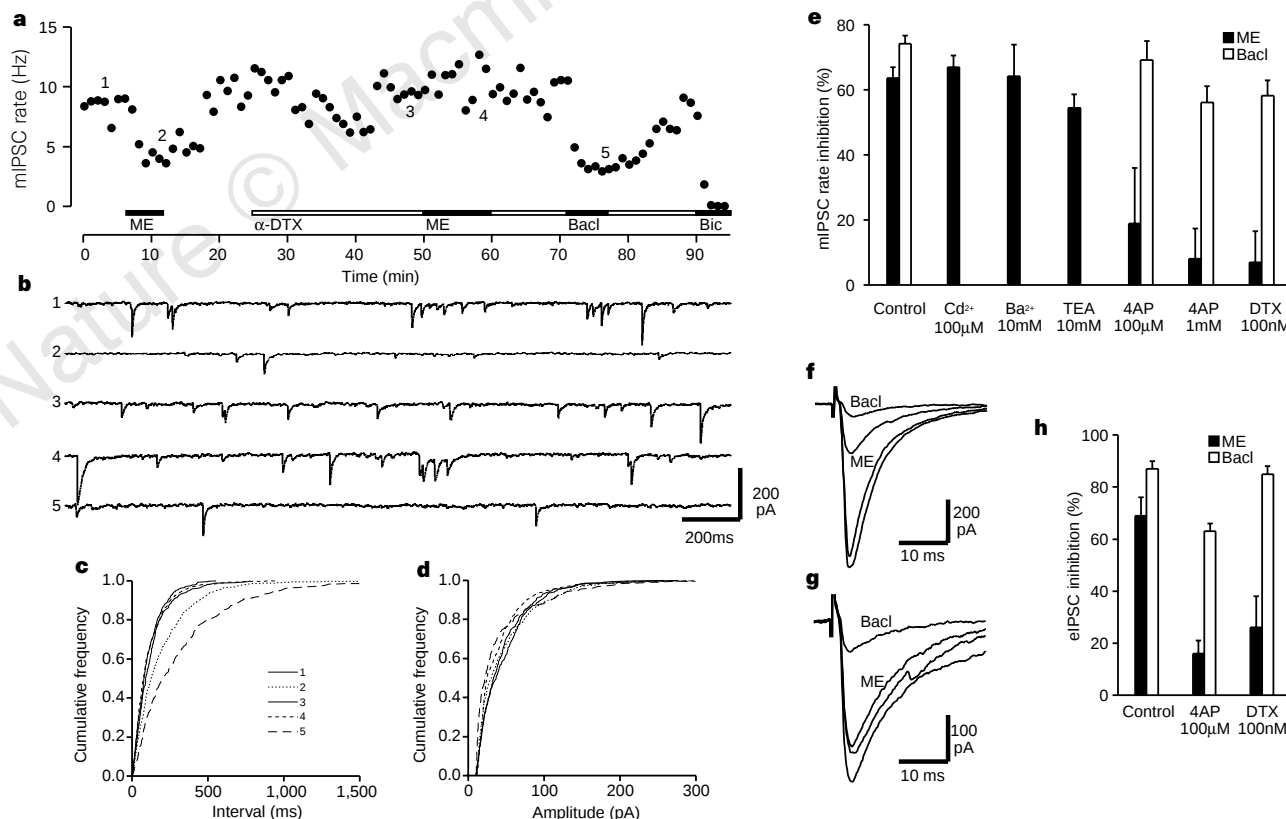


Figure 1 Effect of K⁺ conductance blockade on GABAergic synaptic transmission. **a**, Time course of mIPSC rate during superfusion of ME (10 μ M), then ME and baclofen (Bacl, 10 μ M) in the continuous presence of dendrotoxin (α -DTX, 100 nM), and the GABA_A antagonist bicuculline (Bic, 30 μ M). **b**, Raw current traces at the times indicated in **a**. Cumulative distribution plots of mIPSC: **c**, inter-event interval, and **d**, amplitude at the times indicated in **a**. **e**, Pooled percentage

inhibition of mIPSC rate by ME (black bars) and baclofen (white bars) in the presence of Ca²⁺ and K⁺ conductance blockers. Averaged traces of eIPSCs before, during and after application of ME and baclofen in the **f**, absence, and **g**, presence of dendrotoxin (100 nM). **h**, Pooled percentage inhibition of eIPSC amplitude by ME (black bars) and baclofen (white bars).

satetraenoic acids (HPETEs) by 5- and 12-lipoxygenase, which are then reduced to hydroxyeicosatetraenoic acids (HETEs) or are transformed into leukotrienes and hepxilins (Fig. 2g)¹⁸. The 12-lipoxygenase metabolites ($\pm$)12-HETE (5 μ M; $37 \pm 7\%$ inhibition; $n = 6$) and 8-hydroxy-11,12-epoxy-eicosatrienoic acid (hepxilin A₃; 1 μ M; $32 \pm 7\%$ inhibition; $n = 5$) reduced mIPSC frequency (Fig. 2e) without any effect on mIPSC amplitude ($0 \pm 5\%$ inhibition; $n = 11$ pooled). The finding that distinct 12-lipoxygenase metabolites are active is supported by the observation that the cytochrome P450 inhibitor proadifen (30 μ M; $n = 6$), which prevents conversion of 12-HPETE to hepxilins¹⁶, only partially blocked the ME-induced reduction in mIPSC frequency (Fig. 2f). The 5-lipoxygenase metabolites ($\pm$)5-HETE (5 μ M; $n = 4$) and leukotriene C₄ (3 μ M; $n = 3$) did not significantly reduce mIPSC frequency ($-10 \pm 5\%$ inhibition; Fig. 2e).

Cyclooxygenase inhibitors produce analgesia when microinjected into PAG but the mechanisms involved are unknown⁵. We

considered the possibility that blockade of cyclooxygenase and 5-lipoxygenase might lead to shunting of arachidonic acid metabolism towards the 12-lipoxygenase pathway, thereby enhancing opioid inhibition of GABAergic neurotransmission. Although morphine, a partial agonist of the μ -receptor, at 10 μ M had little effect on mIPSC frequency ($9 \pm 5\%$ inhibition; $n = 6$), it significantly reduced mIPSC frequency in the presence of the cyclooxygenase inhibitors indomethacin (10 μ M; $n = 8$) and aspirin (30 μ M; $n = 7$) (Fig. 3a, b). Morphine (10 μ M) inhibited eIPSCs more in the presence of indomethacin (10 μ M, $54 \pm 8\%$ inhibition; $n = 5$) than in its absence ($24 \pm 6\%$; $n = 8$). Furthermore, indomethacin produced a threefold increase in the potency of the μ -receptor agonist DAMGO, shifting the half-maximal inhibitory concentration (IC₅₀) for mIPSC frequency inhibition from 96 to 32 nM (Fig. 3b). The effect of morphine (10 μ M) on the mIPSC frequency was also enhanced in the presence of the 5-lipoxygenase inhibitor caffeic acid (30 μ M; $n = 5$, Fig. 3b).

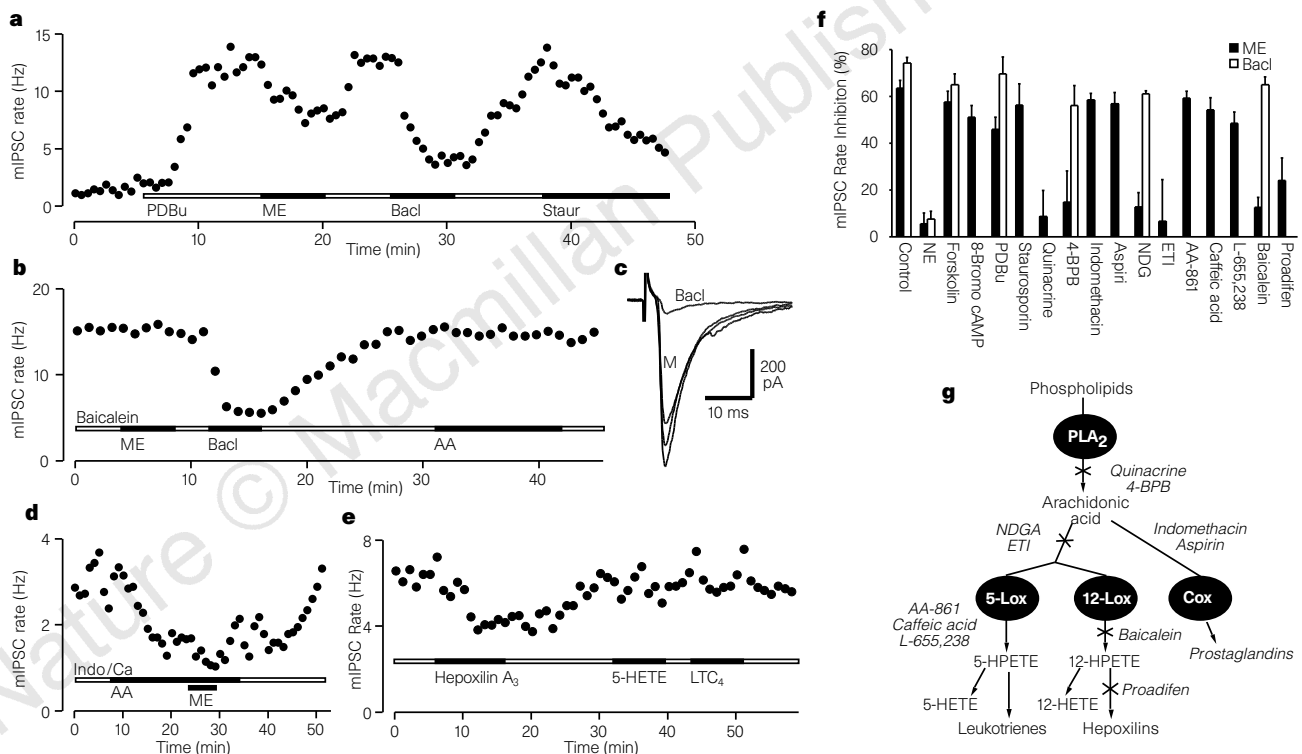


Figure 2 Effect of second messenger modulation on GABAergic synaptic transmission. **a**, Time course of mIPSC rate during superfusion of PDBu (1 μ M), plus ME (10 μ M), baclofen (Bac; 10 μ M), then staurosporin (Staur; 1 μ M). **b**, Time course of mIPSC rate during superfusion of ME, baclofen and arachidonic acid (AA; 150 μ M) in the continuous presence of baicalein (3 μ M). **c**, Averaged traces of eIPSCs before, during and after application of ME and baclofen in presence of baicalein. **d**, Time course of mIPSC rate during superfusion of arachidonic acid

plus ME in the continuous presence of both indomethacin and caffeic acid (Indo/Caff; 10 μ M and 30 μ M). **e**, Time course of mIPSC rate during superfusion of hepxilin A₃ (1 μ M), 5-HETE (5 μ M) and leukotriene C₄ (LTC₄; 3 μ M). **f**, Pooled percentage inhibition of mIPSC rate by ME (black bars) and baclofen (white bars) in the presence of second messenger pathway modulators. **g**, Arachidonic acid metabolism (inhibitors are shown in italics), with crosses indicating the effective inhibitors.

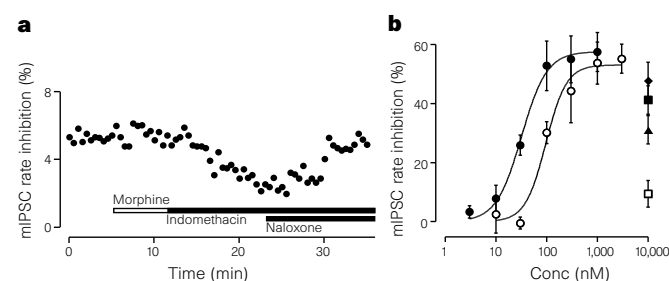


Figure 3 Potentiation of opioid inhibition of GABAergic synaptic transmission by cyclooxygenase and 5-lipoxygenase blockade. **a**, Time course of mIPSC rate during superfusion of morphine (10 μ M), plus indomethacin (10 μ M), then indomethacin plus naloxone (1 μ M). **b**, Concentration-response relationship for percentage mIPSC rate inhibition by DAMGO in the absence (○) and presence (●) of indomethacin (10 μ M); and by morphine in the absence (□) and presence (■) of indomethacin (10 μ M), aspirin (30 μ M) and caffeic acid (30 μ M). Each point shows the mean ($\pm$ s.e.m.) of responses of 4–6 different neurons. A logistic function was fitted to the DAMGO curves to determine the EC₅₀.

Our results show that μ -opioid receptors couple to a voltage-dependent K^+ conductance in GABAergic terminals through the PLA₂/arachidonic acid/12-lipoxygenase cascade system. It is likely that 12-HETE, heptaxilin A₃, and possibly other 12-lipoxygenase metabolites^{16,18} directly or indirectly modulate this presynaptic K^+ conductance. Whereas 5-lipoxygenase-mediated transmitter actions on mammalian postsynaptic K^+ conductances have been described^{17,20,21}, 12-lipoxygenase-mediated transmitter action on a presynaptic K^+ conductance has only been demonstrated in invertebrates²².

These findings also suggest that the actions of μ -receptor agonists in the PAG are potentiated by inhibitors of cyclooxygenase and 5-lipoxygenase. Microinjections of cyclooxygenase inhibitors into the PAG produce analgesia⁵ and these non-steroidal anti-inflammatory drugs are thought to potentiate the analgesic actions of opioid agonists⁶. Possible analgesic activity of 5-lipoxygenase inhibitors and their interactions with opioids have, to our knowledge, not been explored. □

Methods

Midbrain PAG slices (200–300 μ m) were cut from 11–30-day-old Sprague-Dawley rats and were maintained at 34 °C in a submerged chamber containing artificial cerebrospinal fluid (ACSF) equilibrated with 95% O₂ and 5% CO₂, and were later transferred to a superfusing chamber (32 °C) for recording⁴. The ACSF contained (in mM): NaCl, 126; KCl, 2.5; NaH₂PO₄, 1.4; MgCl₂, 1.2; CaCl₂, 2.4; glucose, 11; NaHCO₃, 25. PAG neurons were visualized using infrared Nomarski optics and whole-cell voltage clamp recordings (holding potential, –70 mV) were made using patch electrodes (2–5 M Ω) containing (in mM): CsCl, 140; EGTA, 10; HEPES, 5; CaCl₂, 2; Mg-ATP, 2 (pH 7.3, osmolarity 270–290 mosmol l^{–1}). Series resistance (<12 M Ω) was compensated by 80% and continuously monitored during experiments. Liquid junction potentials of –4 mV were corrected.

eIPSCs were elicited by bipolar tungsten-stimulating electrodes placed near the recording electrode (rate, 0.03 Hz; stimuli: 5–50 V, 20–400 μ s) in the presence of CNQX (3 μ M), and four consecutive responses were averaged (pClamp, Axon). Spontaneous mIPSCs or mEPSCs were obtained in the presence of TTX (0.3 μ M) and CNQX (3 μ M) or bicuculline (30 μ M), filtered at 2 or 5 kHz and recorded on video tape (using a SONY PCM501). Miniature PSCs were sampled at 5 or 10 kHz (Fetchex) for later off-line analysis (Axograph, Axon)⁴. Events were automatically detected by selecting events in which non-consecutive 1- or 0.5-ms segments exceeded a preset threshold (set to 6–15 pA for a rejection rate of at least 10%). Events were ranked by amplitude and interevent interval to construct cumulative probability distributions. Time–mIPSC rate plots were constructed by counting the number of events above a preset threshold (6–15 pA) in 30–60 s epochs (Superscope, GW Instruments). All data are expressed as means $\pm$ s.e.m.

Stock solutions of all drugs were diluted to working concentrations using ACSF immediately before use and applied by superfusion. All PLA₂ pathway inhibitors, except indomethacin, were superfused for at least one hour before the experiment. Arachidonic acid methyl ester was dissolved in dimethyl sulphoxide (DMSO) and lipoxygenase metabolites in ethanol under nitrogen, then stored at –70 °C. Lipoxygenase metabolites were partly dried under nitrogen in the dark immediately before use. AA and the metabolites were dissolved by sonication in ACSF containing 30 μ M ascorbic acid, then superfused with 0.1% DMSO and <0.15% ethanol, respectively. Dendrotoxin (α -dendrotoxin and toxin I) was dissolved in ACSF containing 0.1% bovine serum albumin. Quinacrine, 4-BPB, bicuculline methiodide, 8-bromo-cAMP, TEA and 4-AP were dissolved directly in ACSF. PDBu and L-655,238 were superfused with 0.03% ethanol. Other second-messenger pathway modulators and CNQX were superfused with 0.01–0.1% DMSO. Stock solutions of all other drugs were made up in distilled water. ACSF was altered in experiments using cadmium (NaH₂PO₄-free) and barium (NaH₂PO₄-free, 5 mM NaHCO₃, 5 mM HEPES) and the NaCl concentration adjusted to maintain osmolarity at 320 mosmol l^{–1}.

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1. Mansour, A., Fox, C. A., Akil, H. & Watson, S. J. Opioid-receptor mRNA expression in the rat CNS—
anatomical and functional implications. *Trends Neurosci.* **18**, 22–29 (1995).

2. Basbaum, A. I. & Fields, H. L. Endogenous pain control systems: brainstem spinal pathways and endorphin circuitry. *Annu. Rev. Neurosci.* **7**, 309–338 (1984).
3. Capogna, M., Gähwiler, B. H. & Thompson, S. M. Mechanism of μ -opioid receptor-mediated presynaptic inhibition in the rat hippocampus *in vitro*. *J. Physiol. (Lond.)* **470**, 539–558 (1993).
4. Vaughan, C. W. & Christie, M. J. Presynaptic inhibitory action of opioids on synaptic transmission in the rat periaqueductal grey *in vitro*. *J. Physiol. (Lond.)* **498**, 463–472 (1997).
5. Tortorici, V. & Vanegus, H. Anti-nociception induced by systemic or PAG-microinjected lysine-acetylsalicylate in rats. Effects on tail-flick related activity of medullary off- and on-cells. *Eur. J. Neurosci.* **7**, 1857–1865 (1995).
6. Maves, T. J., Pechman, P. S., Meller, S. T. & Gebhart, G. F. Ketorolac potentiates morphine antinociception during visceral nociception in the rat. *Anesthesiology* **80**, 1094–1101 (1994).
7. North, R. A. Opioid actions on membrane ion channels. In *Opioids* (ed. Herz, A.) 773–798 (Springer, Berlin, 1993).
8. Wimpey, T. & Chavkin, C. Opioids activate both an inward rectifier and a novel voltage-gated potassium conductance in the hippocampal formation. *Neuron* **6**, 281–289 (1991).
9. Grissmer, S. et al. Pharmacological characterization of five cloned voltage-gated K⁺ channels, types Kv1.1, 1.2, 1.3, 1.5, and 3.1, stably expressed in mammalian cell lines. *Mol. Pharmacol.* **45**, 1227–1234 (1994).
10. Vaughan, C. W., Ingram, S. L. & Christie, M. J. Actions of the ORL1 receptor ligand, nociceptin, on membrane properties of rat periaqueductal gray neurons *in vitro*. *J. Neurosci.* **17**, 996–1003 (1997).
11. Simmons, M. L. & Chavkin, C. κ -Opioid receptor activation of a dendrotoxin-sensitive potassium channel mediates presynaptic inhibition of mossy fiber neurotransmitter release. *Mol. Pharmacol.* **50**, 80–85 (1996).
12. Shapiro, M., Wollmuth, L. & Hille, B. Modulation of Ca²⁺ channels by PTX-sensitive G-proteins is blocked by N-ethylmaleimide in rat sympathetic neurons. *J. Neurosci.* **14**, 7109–7116 (1994).
13. Capogna, M., Gähwiler, B. H. & Thompson, S. M. Presynaptic enhancement of inhibitory synaptic transmission in the rat hippocampus *in vitro*. *J. Neurosci.* **15**, 1249–1260 (1995).
14. Fukuda, K., Kato, S., Morikawa, H., Shoda, T. & Mori, K. Functional coupling of the δ -, μ -, and κ -opioid receptors to mitogen-activated protein kinase and arachidonate release in chinese hamster ovary cells. *J. Neurochem.* **67**, 1309–1316 (1996).
15. Blackwell, G. J. & Flower, R. J. Inhibition of phospholipase. *Brit. Med. Bull.* **39**, 260–264 (1983).
16. Piomelli, D. & Greengard, P. Lipoxygenase metabolites of arachidonic acid in neuronal transmembrane signalling. *Trends Pharmacol. Sci.* **11**, 367–373 (1990).
17. Duerson, K., White, R. E., Jiang, F., Schonbrunn, A. & Armstrong, D. L. Somatostatin stimulates BK_{Ca} channels in rat pituitary tumor cells through lipoxygenase metabolites of arachidonic acid. *Neuropharmacology* **35**, 949–961 (1996).
18. Shimizu, T. & Wolfe, L. S. Arachidonic acid cascade and signal transduction. *J. Neurochem.* **55**, 1–15 (1990).
19. Evans, J. F. et al. 5-Lipoxygenase-activating protein is the target of a quinole class of leukotriene synthesis inhibitors. *Mol. Pharmacol.* **40**, 22–27 (1991).
20. Kurachi, Y. et al. α -Adrenergic activation of the muscarinic K⁺ channel is mediated by arachidonic acid metabolites. *Pflügers Arch.* **414**, 102–104 (1989).
21. Schweitzer, P., Madamba, S. & Siggins, G. R. Arachidonic acid metabolites as mediators of somatostatin-induced increase of neuronal M-current. *Nature* **346**, 464–467 (1990).
22. Piomelli, D. et al. Lipoxygenase metabolites of arachidonic acid as second messengers for presynaptic inhibition of Aplysia sensory cells. *Nature* **328**, 38–43 (1987).

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A Schwann cell mitogen accompanying regeneration of motor neurons

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Motor neurons are the only adult mammalian neurons of the central nervous system to regenerate following injury¹. This ability is dependent on the environment of the peripheral nerve and an intrinsic capacity of motor neurons for regrowth². We report here the identification, using a technique known as messenger RNA differential display³, of an extracellular signalling molecule, previously described as the pancreatic secreted protein Reg-2 (ref. 4), that is expressed solely in regenerating and developing rat motor and sensory neurons. Axon-stimulated Schwann cell proliferation is necessary for successful regeneration^{5,6}, and we show that Reg-2 is a potent Schwann cell mitogen *in vitro*. *In vivo*, Reg-2 protein is transported along regrowing axons and

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inhibition of Reg-2 signalling significantly retards the regeneration of Reg-2-containing axons. During development, Reg-2 production by motor and sensory neurons is regulated by contact with peripheral targets. Strong candidates for peripheral factors regulating Reg-2 production are cytokines of the LIF/CNTF family, because Reg-2 is not expressed in developing motor or sensory neurons of mice carrying a targeted disruption of the LIF receptor gene, a common component of the receptor complexes for all of the LIF/CNTF family⁷.

Reg-2 is a secreted protein of relative molecular mass 16,000 (M_r 16K) which belongs to a family of similar proteins found in the pancreas and gastrointestinal tract under normal and pathological conditions^{4,8,9}. A closely related protein, Reg-1, is an islet cell mitogen and is expressed in developing and Alzheimer's disease-affected human cerebral cortex^{10,11}. Reg-2 is not normally expressed in the adult rat central or peripheral nervous system. Within 24 h of crushing the sciatic nerve, all motor neurons and a subpopulation of sensory neurons express high levels of Reg-2 mRNA and protein (Figs 1a, b, c and 2a, b) and continue to do so for the period of regeneration. The expression of Reg-2 is specific to regenerating motor and sensory neurons, as it is not expressed following a variety of other neuronal lesions, including mechanical and ischaemic cerebral cortex injuries and excitotoxic lesions of the basal ganglia (F.J.L. and S.P.H., unpublished observations). Nerve crush and injection of the retrograde tracer Fluorogold into the proximal nerve indicated that Reg-2 is expressed by virtually all regenerating sciatic nerve motor neurons (>95%; Fig. 2a, b), but only by a population of medium- to large-sized sensory neurons (~10%; average cell diameter, $39.7 \pm 0.8 \mu\text{m}$). Retrograde tracing of muscle afferents by injection of Fluorogold into muscle and double-labelling with Reg-2, or double-labelling of sensory neurons for the neuropeptide calcitonin gene-related peptide (CGRP) and Reg-2,

three-to-seven days after sciatic nerve crush indicated that Reg-2-containing sensory neurons do not project to muscle and that only a few (<4%) co-express CGRP (data not shown). Reg-2 protein is orthogradely transported along growing motor and sensory axons, as demonstrated by the accumulation of Reg-2 protein proximal to the site of a peripheral nerve ligation (Fig. 2c). *In situ* hybridization with an oligonucleotide probe specific for Reg-2 mRNA demonstrated that Reg-2 is not transcribed in the resting or regenerating peripheral nerve (data not shown). During rat development, Reg-2 is not expressed before embryonic day 15 (see below). At this stage, Reg-2 is expressed by motor and sensory neurons but by no other cell type, and is expressed in some motor and sensory neurons until the fifth postnatal day.

To investigate the possible role of this protein in regeneration *in vivo*, we tested the effect of inhibiting Reg-2 signalling in the sciatic nerve during regeneration. Reg-2 signalling was inhibited *in vivo* by intraneural injection of an anti-Reg-2 polyclonal antiserum that blocks the mitogenic effects of Reg-2 *in vitro*. Four days after nerve crush and antiserum administration, the number of Reg-2-containing axons crossing a point 3 mm from the site of injury was reduced to approximately one-third, compared to Reg-2-containing axons in animals treated with preimmune serum (18.3 ± 1.6 Reg-2-containing axons per section at 3 mm distal from the injury site in control animals, compared to 6.3 ± 1 Reg-2-containing axons per section at 3 mm distal from the injury site in animals treated with anti-Reg-2 antiserum; Fig. 3); this effect was specific for Reg-2-containing axons. The regeneration of axons containing the neuropeptide CGRP was slightly reduced in the same series of experiments (from an average of 116 ± 8 axons per section to 103 ± 13 axons per section following Reg-2 inhibition; Fig. 3), although this did not reach significance ($P = 0.19$). However, CGRP-containing axons are a mixture of motor and sensory axons¹², the motor axons

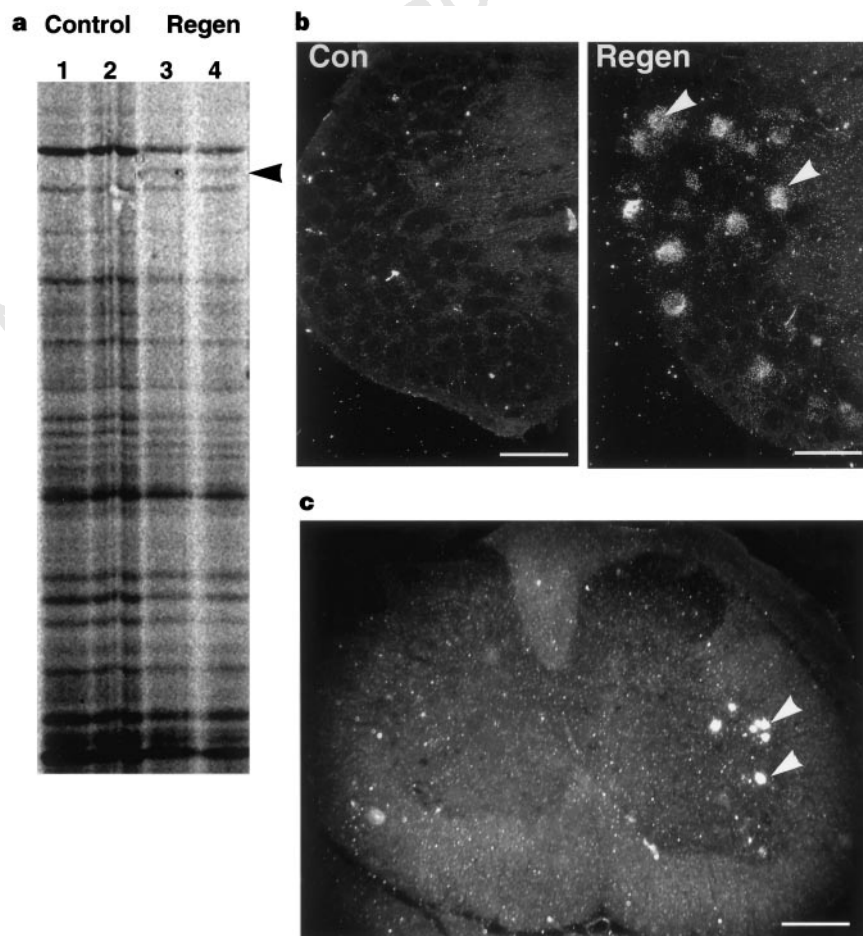


Figure 1 Identification of Reg-2 as a gene expressed in regenerating motor and sensory neurons. **a**, mRNA differential display gel comparing gene expression in resting dorsal root ganglia (DRGs; control, lanes 1 and 2) with that in regenerating DRGs (Regen; lanes 3 and 4). Arrow indicates a 600-bp cDNA expressed solely in regenerating DRGs that encodes Reg-2. **b**, Confirmation by *in situ* hybridization of the induction of Reg-2 expression in regenerating sensory neurons. Arrowheads indicate Reg-2-expressing neurons. **c**, Reg-2 expression in spinal cord motor neurons projecting into the regenerating sciatic nerve (arrowheads) in a coronal section of the lumbar spinal cord. Scale bars: **b**, 100 μm ; **c**, 200 μm .

of which also express Reg-2, whereas most of the CGRP-expressing sensory neurons do not co-express Reg-2 (<4% of CGRP-containing sensory neurons co-express Reg-2; data not shown).

Given the effects of blocking Reg-2 signalling *in vivo* and the temporal expression of Reg-2 during development, we examined the effect of Reg-2 on Schwann cell proliferation *in vitro*. Recombinant Reg-2 acts as a weak mitogen for Schwann cells *in vitro* (Fig. 4), as do other known Schwann cell mitogens, such as basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF)¹³. However, in the presence of forskolin, an activator of adenylyl cyclase, Reg-2 is strongly mitogenic for Schwann cells (Fig. 4). This

is a well described phenomenon for other known Schwann cell mitogens, including the glial growth factors (GGFs), bFGF and PDGF, and is attributed to a cyclic AMP-induced increase in receptor expression for those factors^{6,14}.

To test the hypothesis that expression of Reg-2 in developing motor neurons is regulated by peripherally derived factors, we investigated the effect of axotomy on Reg-2 expression in the neonatal period. Neonatal rodent motor neurons are developing neurons, which do not assume an adult phenotype until approximately the fifth postnatal day¹⁵. Unlike their adult counterparts, neonatal motor neurons are particularly sensitive to peripheral nerve axotomy, with as many as 90% of lumbar motor neurons projecting into the sciatic nerve undergoing apoptosis in the week following sciatic nerve injury of the first postnatal day¹⁵. This sensitivity to axotomy has been attributed to the interruption of retrograde transport of peripherally derived neurotrophic factors, as survival of neonatal motor neurons following axotomy can be increased to a varying degree by administration of a range of factors, including BDNF¹⁶ and CNTF¹⁷. Within 24 h of peripheral nerve axotomy in newborn rat pups, constitutive Reg-2 protein levels in sciatic motor neurons are markedly reduced compared with those in uninjured motor neurons of the opposite side of the spinal cord ($n = 5$, Fig. 5a). This reduction in Reg-2 expression precedes motor neuron cell death, approximately half of which occurs in the first four days after axotomy¹⁸. The rapid downregulation of Reg-2 protein following axotomy in the neonate indicates that Reg-2 expression in developing motor neurons is regulated by contact with peripheral targets.

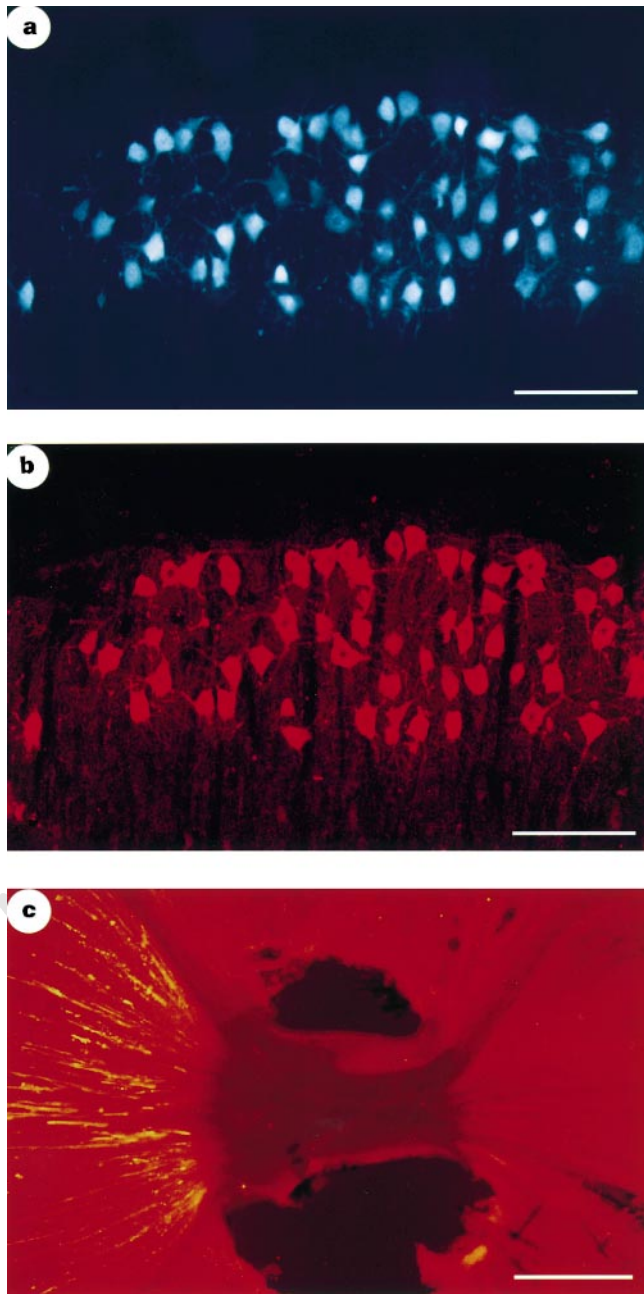


Figure 2 Reg-2 protein localization in regenerating motor neurons. **a**, Longitudinal section through the pool of regenerating sciatic nerve motor neurons, retrogradely labelled with Fluorogold. **b**, Fluorescent micrograph of Reg-2 immunoreactivity in the same section. Almost all regenerating motor neurons express Reg-2. **c**, Reg-2 is orthogradely transported along regenerating axons in the peripheral nerve, as demonstrated by Reg-2 immunoreactivity in the peripheral axons proximal, but not distal, to the site of peripheral nerve ligation. Scale bars, 250 μ m.

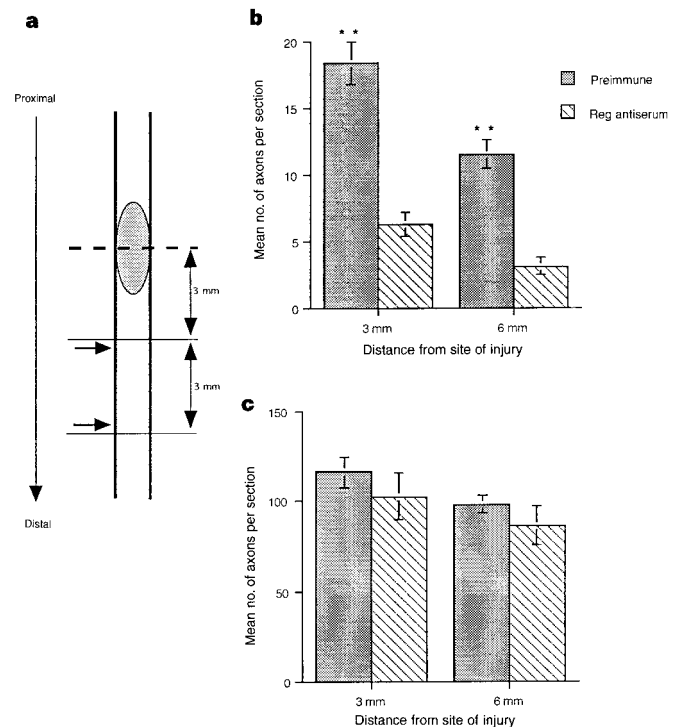


Figure 3 *In vivo* inhibition of Reg-2 retards regeneration. **a**, Experimental procedure. Antisera were injected into the shaded area; the dashed line indicates the site of nerve crush. Arrows indicate the two points at which we counted the number of axons containing either CGRP or Reg-2 and crossing each point in a longitudinal section. A measure of the number of axons crossing the 3-mm and 6-mm points was obtained by counting all of the labelled axonal profiles in focus in a single, optimal focal plane in four sections from each nerve. **b**, Reduction in the number of Reg-2-containing axons crossing two points distal to the site of injury following inhibition of Reg-2 activity *in vivo*. **, $P < 0.001$ at each distance (Student's t -test). **c**, Effects of inhibiting Reg-2 activity on the regeneration of CGRP-containing axons; $P \approx 0.2$ at each distance.

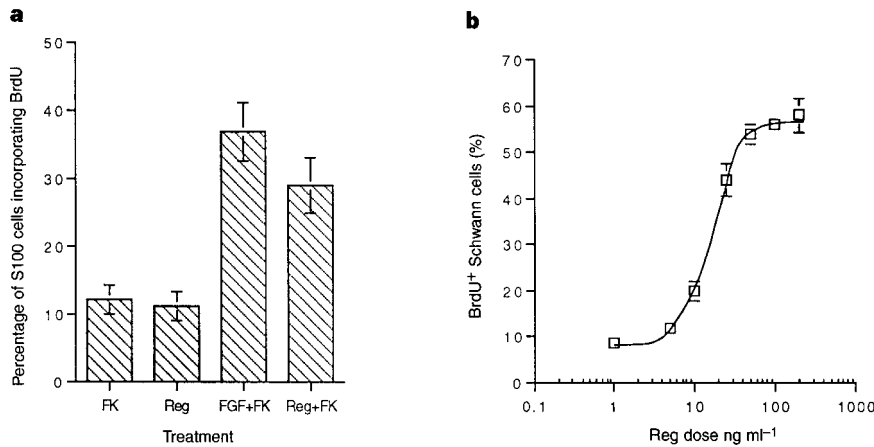


Figure 4 Reg-2 is a Schwann-cell mitogen *in vitro*. **a**, Percentage of Schwann cells double-labelled for BrdU incorporation (an indicator of DNA synthesis) and S100 (a Schwann cell marker) in the 24 h following each treatment. Bars indicate the mean percentage of Schwann cells double-labelled for BrdU averaged over three separate experiments, error bars indicate s.e.m. FK, forskolin; FGF, basic fibroblast growth factor; Reg, recombinant Reg-2 protein. **b**, Dose-response curve for the mitogenic effect of Reg-2 on Schwann cells in the presence of 10 µm forskolin. Error bars indicate s.e.m.

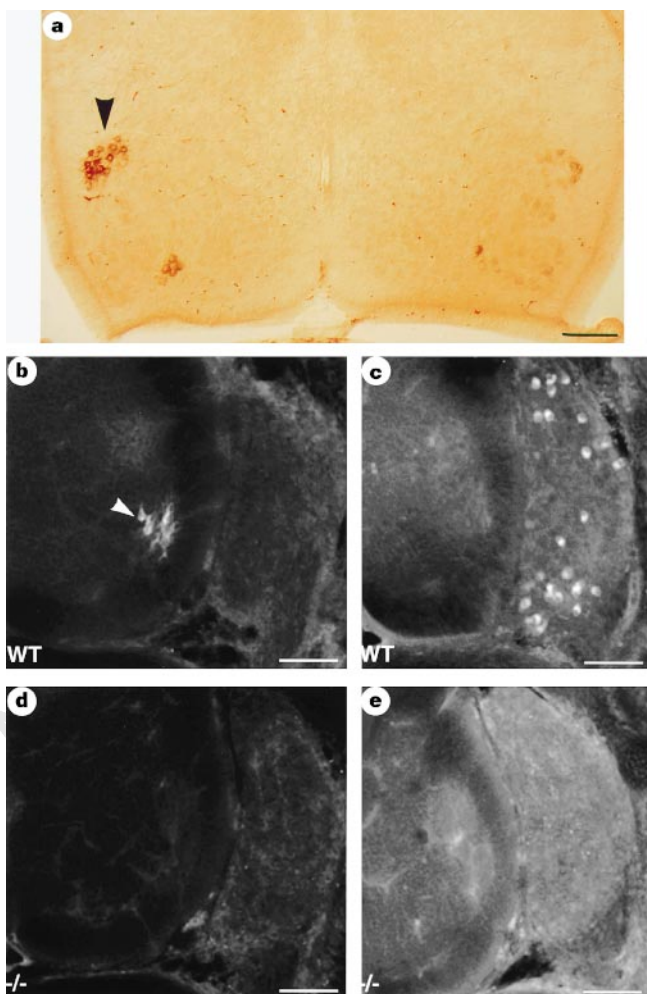


Figure 5 Reg-2 expression in developing neurons is regulated by peripherally derived neurotrophic factors. **a**, Reg-2 protein expression in the neonatal spinal cord 24 h after unilateral sciatic nerve axotomy. Reg-2 is constitutively expressed in the pool of motor neurons projecting into the sciatic nerve (arrowhead). On the site of axotomy, Reg-2 expression has decreased to low levels. **b–e**, Confocal scanning laser photomicrographs of Reg-2 protein expression in the lumbar spinal cords of wild-type (WT) and LIFR-mutant mouse embryo littermates. **b, c**, Reg-2 in motor neurons (**b**; indicated by arrowhead) and DRG (**c**) of E15 WT spinal cord. **d, e**, Absence of Reg-2 expression in the spinal cord (**d**) and DRG (**e**) of E15 LIFR^{-/-} mouse embryos. Expression was studied in three WT, three LIFR-mutant heterozygotes and three LIFR-mutant homozygotes. Scale bars: **a**, 100 µm; **b, d**, 150 µm; **c, e**, 120 µm.

Strong candidates for factors produced by peripheral targets to regulate expression of Reg-2 are cytokines of the LIF/CNTF family. Studies on the control of Reg-2 transcription in the pancreas have identified two interleukin-6 response elements (IL-6REs; STAT-binding elements¹⁹) in the promoter region of the rat Reg-2 gene²⁰. Interleukin-6-related neurotrophic cytokines of the LIF/CNTF family, including leukaemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF) and cardiotrophin-1 (CT-1), are expressed in developing and adult peripheral tissues^{17,21,22}. All of the members of this subfamily of cytokines signal through receptor complexes containing the common transmembrane units, the IL-6 receptor gp130 and the LIF receptor (LIFR), and activate the intracellular JAK–STAT signalling pathway^{19,23}. Targeted disruption of the LIFR gene removes a common component of the LIF, CNTF and CT-1 receptors and thus inhibits signalling by all of those factors during development⁷. Therefore, we analysed E15 mouse embryos homozygous for a disrupted LIFR gene⁷ and found that Reg-2 is not expressed by developing motor and sensory neurons in mutant animals (Fig. 5b–e). Reg-2 is expressed in both of those cell types in both wild-type littermates and embryos heterozygous for the disrupted gene (Fig. 5). Thus, Reg-2 expression in developing motor and sensory neurons is dependent on cytokines of the LIF family acting through a receptor complex containing LIFR.

Schwann cell dedifferentiation and proliferation are essential for neural regeneration in the adult peripheral nervous system^{5,6}. The classic Schwann cell mitogens, the glial growth factors/neuregulins, are constitutively expressed by motor and sensory neurons in early development and adulthood²⁴ but are not upregulated during regeneration²⁵. In contrast, Reg-2 is only produced during periods of Schwann cell proliferation, that is, in the perinatal period and during regeneration. We have shown that this Schwann cell mitogen is necessary for regeneration of Reg-2-containing axons, a large proportion of which are motor axons. Given the regulation of Reg-2 production during development by peripherally derived factors, including cytokines of the LIF/CNTF family, we propose that Reg-2 is an essential component of the neuron–glia interactions underlying development and regeneration of mammalian motor neurons. □

Methods

In vivo regeneration. All surgery was carried out according to UK Home Office guidelines for the ethical treatment of animals. Sciatic nerve crush, tissue fixation and tissue recovery were carried out as described²⁶. LIFR mutant and wild-type mouse embryos were bred and genotyped as described⁷. Regenerating motor neurons were retrogradely labelled by injection of Fluorogold (5 µl of a 7% solution in PBS) into the proximal stump of the sciatic nerve following axotomy. Intraneural injection of either preimmune or anti-Reg-2 antisera was carried out immediately before sciatic nerve crush by injection of 5 µl

polyclonal antiserum, raised in rabbits against the whole recombinant protein, into the crush site. Four days later, animals ($n = 4$ for each treatment) were killed and fixed as described. Tissue from each experiment was coded before sectioning and all counts were performed blind.

mRNA differential display. Differential display PCR on total cellular RNA extracted from control and regenerating dorsal root ganglia was carried out as described³. The sequences of cloned cDNAs were compared to the GenBank non-redundant and EST databases using the BLAST algorithm²⁷.

In situ hybridization. Oligonucleotides 45 bases in length complementary to the sequence of rat Reg-2 isolated in this screen were synthesized in-house at the LMB core facility (sequence: TGG GAG CCT TTG GGG CAA CTA ATG CGT GCA GAG GGT ATT TTC TTC). The oligonucleotide was 3'-tailed with [α -³⁵S]dATP and hybridized to 15- μ m sections of developing, lesioned and adult tissues as described²⁸.

Immunohistochemistry. Polyclonal antisera or monoclonal antibodies against Reg-2, CGRP (Affiniti), S100 (Dako) and BrdU (Boehringer) were applied to tissue sections or fixed cells at 4°C for 48 h. Antibody detection and visualization have been described²⁹. Controls for each antibody consisted of a preabsorption with recombinant Reg-2 for the Reg-2 polyclonal antiserum and omission of the first antibody or antiserum from the process for all others. For the *in vivo* Reg-2 inhibition experiments, a control experiment to detect any residual anti-Reg-2 antiserum within the tissue from the original procedure was carried out by omitting the Reg-2 polyclonal antiserum and directly applying the appropriate secondary antibody and carrying out the standard detection procedures.

Schwann cell culture. Primary cultures of dissociated Schwann cells were prepared from the sciatic nerve of neonatal rats and maintained in Dulbecco's minimal essential medium plus N2 supplement as described²⁹. Basic FGF (50 ng ml⁻¹; Sigma), recombinant Reg-2 (2–100 ng ml⁻¹; produced as described³⁰) and forskolin (10 μ M; Sigma) were added to cultures 24 h after plating, together with 10 μ M bromodeoxyuridine (BrdU). 24 h later, cultures were fixed and cells were processed for BrdU and S100 immunohistochemistry. The proportion of S100-positive cells that were also BrdU-positive was calculated in three separate experiments. The capacity of the anti-Reg-2 antiserum to inhibit Reg-2 signalling *in vitro* was assessed by measuring the ability of a range of concentrations of antiserum to attenuate the mitogenic effect on Schwann cells of a fixed dose of Reg-2 (30 ng ml⁻¹).

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1. Fawcett, J. W. & Keynes, R. J. Peripheral nerve regeneration. *Annu. Rev. Neurosci.* **13**, 43–60 (1990).
2. Scherer, S. S. & Salzer, J. L. In *Glial Cell Development* (eds Jessen, K. R. & Richardson, W. D.) 165–196 (BIOS, Oxford, 1996).
3. Liang, P. *et al.* Differential display using one-base anchored oligo-dT primers. *Nucleic Acids Res.* **22**, 5763–5764 (1994).
4. Iovanna, J., Orelle, B., Keim, V. & Dagorn, J.-C. Messenger RNA sequence and expression of rat pancreatitis-associated protein, a lectin-related protein overexpressed during acute experimental pancreatitis. *J. Biol. Chem.* **266**, 25664–25669 (1991).
5. Hall, S. M. The effect of inhibiting Schwann cell mitosis on the re-innervation of acellular autografts in the peripheral nervous system of the mouse. *Neuropath. Appl. Neurobiol.* **12**, 401–414 (1986).
6. Reynolds, M. L. & Woolf, C. J. Reciprocal Schwann cell–axon interactions. *Curr. Opin. Neurobiol.* **3**, 683–693 (1993).
7. Li, M., Sendtner, M. & Smith, A. Essential function of LIF receptor in motor neurons. *Nature* **378**, 724–727 (1995).
8. Frigerio, J.-M., Dusetti, N. J., Keim, V., Dagorn, J.-C. & Iovanna, J. L. Identification of a second rat pancreatitis-associated protein. Messenger RNA cloning, gene structure, and expression during acute pancreatitis. *Biochemistry* **32**, 9236–9241 (1993).
9. Frigerio, J. M., Dusetti, N. J., Garrido, P., Dagorn, J.-C. & Iovanna, J.-L. The pancreatitis-associated protein-III (PAP-III), a new member of the PAP gene family. *Biochim. Biophys. Acta* **1216**, 329–331 (1993).
10. Watanabe, T. *et al.* Pancreatic beta-cell replication and amelioration of surgical diabetes by Reg protein. *Proc. Natl Acad. Sci. USA* **91**, 3589–3592 (1994).
11. de la Monte, S. M., Ozturk, M. & Wands, J. R. Enhanced expression of an exocrine pancreatic protein in Alzheimer's disease and the developing human brain. *J. Clin. Invest.* **86**, 1004–1013 (1990).
12. Dumoulin, F. L., Raivich, G., Streut, W. J. & Kreutzberg, G. W. Differential regulation of calcitonin gene-related peptide (CGRP) in regenerating rat facial nucleus and dorsal root ganglion. *Eur. J. Neurosci.* **3**, 338–342 (1991).
13. Eccleston, P. A. Regulation of Schwann cell proliferation: mechanisms involved in peripheral nerve development. *Exp. Cell Res.* **199**, 1–9 (1992).
14. Weinmaster, G. & Lemke, G. Cell-specific cyclic AMP-mediated induction of the PDGF receptor. *EMBO J.* **9**, 915–920 (1990).
15. Greensmith, L. & Vrbová, G. Motoneuron survival: a functional approach. *Trends Neurosci.* **19**, 450–455 (1996).
16. Yan, Q., Elliott, J. & Snider, W. D. Brain-derived neurotrophic factor rescues spinal motor neurons from axotomy-induced cell death. *Nature* **360**, 753–759 (1992).
17. Sendtner, M., Kreutzberg, G. W. & Thoenen, H. Ciliary neurotrophic factor prevents the degeneration of motor neurons after axotomy. *Nature* **345**, 440–441 (1990).
18. Schmalbruch, H. & Rosenthal, A. Neurotrophin-4/5 postpones the death of injured spinal motoneurons in newborn rats. *Brain Res.* **700**, 254–260 (1995).
19. Schindler, C. & Darnell Jr, J. E. Transcriptional responses to polypeptide ligands: the JAK-STAT pathway. *Annu. Rev. Biochem.* **64**, 621–651 (1995).

20. Dusetti, N. J., Ortiz, E. M., Mallo, G. V., Dagorn, J.-C. & Iovanna, J. L. Pancreatitis-associated protein (PAP I), an acute phase protein induced by cytokines. *J. Biol. Chem.* **270**, 22417–22421 (1995).
21. Banner, L. R. & Patterson, P. H. Major changes in the expression of the mRNAs for cholinergic differentiation factor/leukaemia inhibitory factor and its receptor after injury to adult peripheral nerves and ganglia. *Proc. Natl Acad. Sci. USA* **91**, 7109–7113 (1994).
22. Pennica, D. *et al.* Cardiotrophin-1, a cytokine present in embryonic muscle, supports long-term survival of spinal motoneurons. *Neuron* **17**, 63–74 (1996).
23. Stahl, N. *et al.* Association and activation of Jak-Tyk kinases by CNTF-LIF-OSM-IL-6 β receptor components. *Science* **263**, 92–95 (1994).
24. Gassmann, M. & Lemke, G. Neuregulins and neuregulin receptors in neural development. *Curr. Opin. Neurobiol.* **7**, 87–92 (1997).
25. Carroll, S. L., Miller, M. L., Frohnert, P. W., Kim, S. S. & Corbett, J. A. Expression of neuregulins and their putative receptors, ErbB2 and ErbB3, is induced during Wallerian degeneration. *J. Neurosci.* **17**, 1642–1659 (1997).
26. Jenkins, R. & Hunt, S. P. Long term increases in the levels of c-jun mRNA and Jun protein like immunoreactivity in motor and sensory neurons following axon damage. *Neurosci. Lett.* **129**, 107–111 (1991).
27. Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. Basic local alignment search tool. *J. Mol. Biol.* **215**, 403–410 (1990).
28. Wisden, W., Morris, B. J. & Hunt, S. P. In *Molecular Neurobiology: A Practical Approach* (eds Chad, J. & Wheal, H.) 205–225 (IRL, Oxford, 1991).
29. De Felipe, C. & Hunt, S. P. The differential control of c-jun expression in regenerating sensory neurons and their associated glial cells. *J. Neurosci.* **14**, 2911–2923 (1994).
30. Chakraborty, C. *et al.* Plasma-clearance, tissue uptake and expression of pituitary peptide 23/pancreatitis-associated protein in the rat. *J. Endocrinol.* **145**, 461–469 (1995).

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Role of cytosolic phospholipase A₂ in allergic response and parturition

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Phospholipase A₂ (PLA₂) comprises a superfamily of enzymes that hydrolyse the ester bond of phospholipids at the *sn*-2 position^{1–3}. Among the members of this superfamily, cytosolic PLA₂ has attracted attention because it preferentially hydrolyses arachidonyl phospholipids and is activated by submicromolar concentrations of Ca²⁺ ions and by phosphorylation by mitogen-activated protein kinases (MAP kinases)^{4–8}. Here we investigate the function of cytosolic PLA₂ *in vivo* by using homologous recombination to generate mice deficient in this enzyme. These mice showed a marked decrease in their production of eicosanoids and platelet-activating factor in peritoneal macrophages. Their ovalbumin-induced anaphylactic responses were significantly reduced, as was their bronchial reactivity to methacholine. Female mutant mice failed to deliver offspring, but these could be rescued by administration of a progesterone-receptor antagonist to the mother at term. Considered together with previous findings^{9–15}, our results indicate that cytosolic PLA₂ plays a non-redundant role in allergic responses and reproductive physiology.

We disrupted the cytosolic PLA₂ (cPLA₂) gene by replacing part of an internal exon with a PGK-neo cassette (Fig. 1a). The targeted exon contains the lipase consensus motif, including the Ser 228 residue that is essential for enzyme activity¹⁶. Three recombinant embryonic stem (ES) clones were identified after transformation of 129/Ola mouse-derived E14-1 ES cells with the targeting vector. Cells from two of the clones were injected into C57BL/6J blastocysts

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to produce chimaeric mice. Male chimaeras containing a substantial contribution of 129/Ola cells were bred to C57BL/6J females to produce F₁ 129/B6 offspring containing a targeted cPLA₂ allele. Heterozygous F₁ mice were interbred and their offspring were genotyped by using the polymerase chain reaction (PCR; data not shown) and Southern blot analysis (Fig. 1b). The genotype distribution among F₂ progeny was in accordance with the mendelian rule (*cpla*₂^{+/+}:*cpla*₂^{+/-}:*cpla*₂^{-/-}): 146:278:135 (males); 140:267:130 (females). The absence of cPLA₂ transcript (Fig. 1c) and immuno-reactive cPLA₂ protein (Fig. 1d) in *cpla*₂^{-/-} mice confirmed the disruption of the cPLA₂ gene. The PLA₂ activities observed in the spleen cytosol were 11.3 ± 1.0 and 0.2 ± 0.1 pmol min⁻¹ mg⁻¹ of protein for *cpla*₂^{+/+} and *cpla*₂^{-/-} mice, respectively (mean ± s.e.m.; *n* = 3, *P* = 0.0004, unpaired *t*-test). Furthermore, when peritoneal macrophages were stimulated with Ca²⁺ ionophore, synthesis of prostaglandin E₂ (PGE₂), cysteinyl leukotrienes (Cys-LT) and platelet-activating factor (PAF) were markedly decreased (Fig. 2a–c). A

marked increase in PGE₂ in *cpla*₂^{+/+} cells treated with lipopolysaccharide (LPS) for 12 h was not evident in *cpla*₂^{-/-} cells (Fig. 2a).

cPLA₂-deficient mice developed normally until they were 10 months old. Gross anatomic and light-microscopic examination of the principal organs and tissues (brain, heart, lung, liver, pituitary, thyroid, adrenal, thymus, spleen, oesophagus, stomach, duodenum, jejunum, ileum, caecum, colon, rectum, bone, bone marrow and skin) revealed no significant differences between wild-type and cPLA₂-deficient male mice at 4.5 months of age (data not shown). However, in three of five cPLA₂-deficient mice, a moderate cytoplasmic vacuolation in the tubular epithelium of the kidney was seen. Although renal abnormalities have also been reported in mice deficient in cyclooxygenase (COX)-1 (ref. 12) and COX-2 (refs 13, 14), the cause of the histological change and its significance are unclear. There were no changes in serum biochemical parameters, including blood urea nitrogen and creatinine concentrations (data not shown).

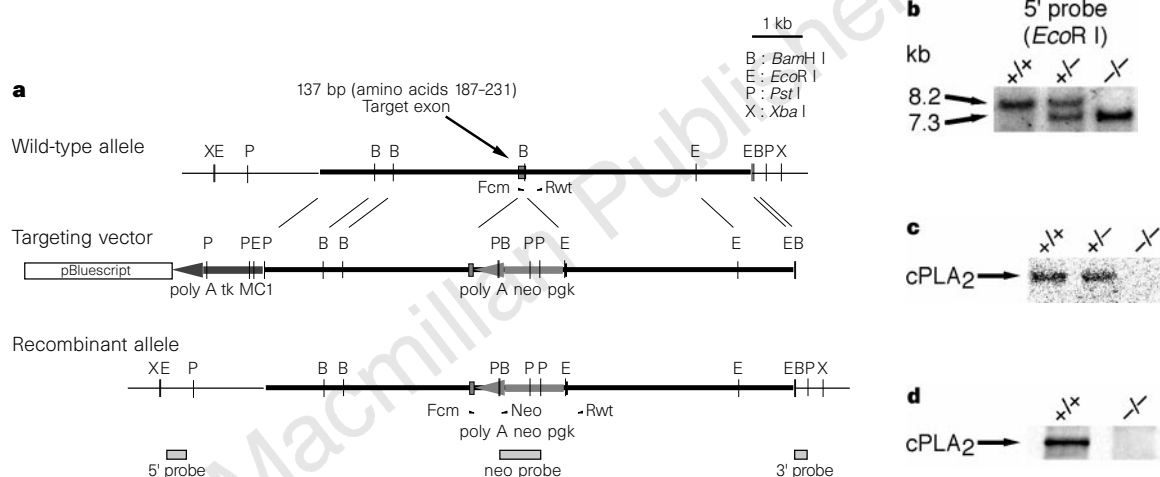


Figure 1 Disruption of the cPLA₂ gene in mice. **a**, Gene-targeting strategy: structure and partial restriction maps of the cPLA₂ gene, targeting construct and predicted recombinant allele. The dotted box and thick lines represent the target exon containing the lipase consensus motif and the parts of introns used as the flanking homologous regions, respectively. A neomycin-resistance gene driven by the PGK promoter (pgk neo poly A) and a herpes simplex virus thymidine kinase gene driven by the MC1 promoter (MC1 tk poly A) are depicted by arrows indicating the direction of transcription. Hatched boxes and wedges (Fcm, Rwt,

Neo) represent the locations of probes used for hybridization analysis and the primers used for PCR, respectively. **b**, Southern blot analysis of tail DNA from *cpla*₂^{+/+}, *cpla*₂^{+/-} and *cpla*₂^{-/-} mice. The wild-type (8.2 kb) and recombinant (7.3 kb) *Eco*RI fragments were identified using the 5' probe indicated in **a**. **c**, Northern blot analysis of cPLA₂ mRNA expression in *cpla*₂^{+/+}, *cpla*₂^{+/-} and *cpla*₂^{-/-} spleen. cPLA₂ mRNA was identified using a full-length ORF cPLA₂ cDNA probe. **d**, Western blot analysis of *cpla*₂^{+/+} and *cpla*₂^{-/-} spleen cytosol. cPLA₂ protein was identified using an anti-cPLA₂ monoclonal antibody (Santa Cruz).

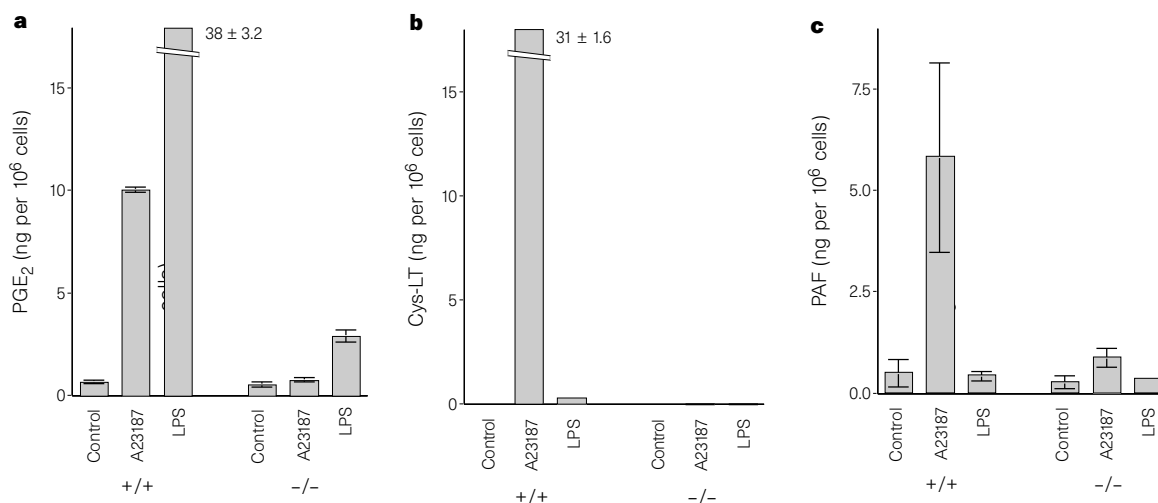


Figure 2 Production of eicosanoids and PAF in peritoneal macrophages. Peritoneal macrophages were isolated from age-matched female mice. Cells were stimulated with A23187 for 10 min or with LPS for 12 h for PGE₂ and Cys-LT

measurements, or for 30 min for PAF measurement. The amount of eicosanoids and PAF produced in the absence of stimulation are labelled as controls. Data are presented as mean ± s.e.m. **a**, PGE₂ (*n* = 3); **b**, Cys-LT (*n* = 3); **c**, PAF (*n* = 2).

Table 1 Summary of mating experiment

Mating pairs (male, female)	Number of females delivered							Offspring* born (survived)	Litter size (average $\pm$ s.d.)
	d18.5	d19.5	d20.5	d21.5	d22.5	d23.5	d24.5		
+ / +, + / + (n = 8)	1	6	1	0	0	0	0	45 (41)	5.6 $\pm$ 3.2
- / -, + / + (n = 8)	2	5	1	0	0	0	0	43 (39)	5.4 $\pm$ 2.7
+ / +, - / - (n = 6)	0	0	2	0	1	2	1	8 (0)	1.3 $\pm$ 0.5
- / -, - / - (n = 7)	0	0	0	4	2	1	0	20 (0)	2.9 $\pm$ 1.6

* Numbers include offspring that died *in utero*; survival was determined during the weaning period.

The type I allergic response (anaphylaxis) underlies the pathogenesis of bronchial asthma, atopic dermatitis and anaphylactic shock. This reaction is triggered by the crosslinking of high-affinity Fc receptors for IgE (Fc ϵ RI) that are expressed on the surface of mast cells and basophils. After antigen stimulation, chemical mediators are rapidly released from these cells, evoking acute airway constriction, hypotension and vascular leakage. In contrast to mediators (histamine, serotonin, and so on), which are stored in granules, lipid mediators such as eicosanoids (prostaglandins and leukotrienes) and PAF are newly synthesized and released. Because antigen stimulation induces a transient Ca²⁺ increase in cells and activates protein kinase cascades, cPLA₂ has been proposed to be involved in the synthesis of these lipid mediators, although without any direct evidence^{17–19}. To determine the role of cPLA₂ in the anaphylactic response, a systemic anaphylaxis was elicited in ovalbumin-immunized mice²⁰ and their pulmonary parameters were measured. We found that there was no significant change in the total lung resistance (R_L) in *cpla2*^{-/-} mice before ovalbumin challenge. Tracheal pressure increased 15 to 20 s after the injection of ovalbumin, and R_L reached a maximum at 2 min (Fig. 3a). The peak R_L values for *cpla2*^{-/-} mice were slightly lower ($184 \pm 27\%$ of baseline, mean $\pm$ s.e.m.; $n = 5$) than those of *cpla2*^{+/+} mice ($251 \pm 39\%$ of baseline, mean $\pm$ s.e.m.; $n = 6$), and *cpla2*^{-/-} mice recovered much faster than *cpla2*^{+/+} mice. The R_L of *cpla2*^{-/-} mice had returned to baseline by 10 min, but the R_L of *cpla2*^{+/+} mice remained in the bronchoconstrictive range, about 50% above the baseline (Fig. 3a). Histologically, wild-type mice showed a prominent folding of bronchial epithelium, narrowing of the airway and alveolar thickening, but these abnormalities were not evident in *cpla2*^{-/-} mice (Fig. 3b). There was a striking difference in the bronchial reactivity to methacholine between the mice. Twenty minutes after elicitation of anaphylaxis, when the tracheal pressure had returned to the basal level, cumulative doses (0.15 – 10 mg ml⁻¹) of methacholine were administered to the mice by inhalation. Sensitized and challenged *cpla2*^{+/+} mice showed increased values of R_L even at the lowest dose (0.15 mg ml⁻¹) of methacholine, demonstrating that bronchial hyperreactivity was induced by the antigen challenge. By contrast, the R_L of *cpla2*^{-/-} mice remained at basal levels at methacholine doses as high as 2.5 mg ml⁻¹, and then increased gradually (Fig. 3c); this sensitivity was essentially identical to that of mice not challenged with ovalbumin. These findings suggest that cPLA₂ is involved in antigen-induced bronchial hyperreactivity. As airway responsiveness to methacholine is markedly decreased in 5-lipoxygenase-deficient mice¹¹, it is possible that cPLA₂ couples with 5-lipoxygenase in the mouse lung under antigen-challenged conditions. We have also observed bronchial hyperreactivity to methacholine in transgenic mice overexpressing PAF receptor²¹.

Prostaglandins (PGs) and PAF are important in reproduction^{21–23}, although it is not clear which type of PLA₂ mainly affects the synthesis of these mediators. After coitus, we monitored the increase in body weight of pregnant female mice. No significant differences were observed when wild-type females were mated, regardless of the genotype of the males. After mating, *cpla2*^{-/-} female mice gained less weight, consistent with a smaller litter size (Table 1). Furthermore, *cpla2*^{-/-} females failed to undergo labour at term (between 18.5 to 20.5 days of pregnancy; Table 1), delivering a few live offspring at ~ 21.5 – 22.5 d gestation, but the pups did not survive (Table 1). Caesarean section of five mice yielded 15 viable

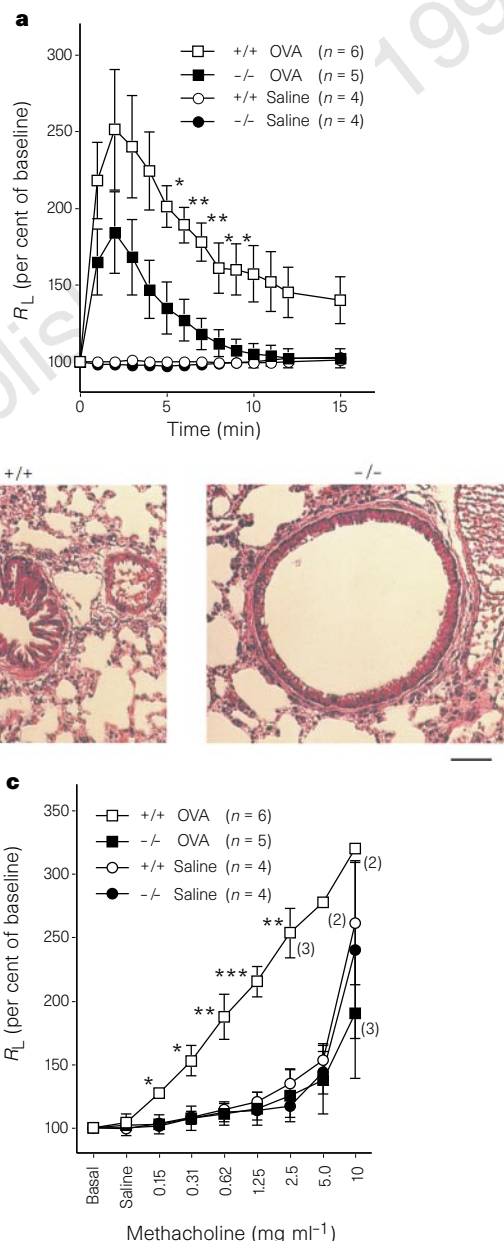


Figure 3 Allergic responses in the bronchopulmonary system. **a**, Comparison of changes in total lung resistance (R_L) between *cpla2*^{+/+} and *cpla2*^{-/-} mice undergoing anaphylaxis in response to injection with ovalbumin (OVA). Data are expressed as mean percentages of baseline $\pm$ s.e.m. The baseline value of R_L corresponds to the mean of values measured over a 5-min period before challenge with OVA. No mice died during the recordings. * $P < 0.05$; ** $P < 0.01$ versus each other group, as determined by analysis of variance (ANOVA) with Scheffe's test for pairwise comparison. **b**, Histology of fixed lung tissue from *cpla2*^{+/+} (left) and *cpla2*^{-/-} (right) mice. Scale bar, 50 μ m. **c**, Comparison of the change in R_L between *cpla2*^{+/+} and *cpla2*^{-/-} mice following methacholine inhalation. Numbers in parentheses are the numbers of animals alive after each dose. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus each other group, as determined by ANOVA with Scheffe's test for pairwise comparisons.

neonates, 11 of which grew to adulthood. Viable neonates could also be obtained by administration of a progesterone receptor antagonist, RU486 (ref. 24): when RU486 was injected subcutaneously (15 mg kg⁻¹) into four *cpla2*^{-/-} mice on day 18.5 of pregnancy, labour was induced within 18 to 24 h and 12 neonates were born, 9 of which grew to adulthood. Administration of vehicle (4% ethanol, 25 µl g⁻¹) failed to induce labour (*n* = 4). COX-deficient mice also showed reproductive abnormalities^{12,13} and mice lacking PGF_{2α} receptor did not come into labour¹⁵. Taken together, our results indicate that cPLA₂ couples with COX to produce PGF_{2α} in female reproductive systems, which in turn causes luteolysis and induction of labour. Further study will be necessary to identify the mechanism by which cPLA₂ affects pregnancy and labour.

In conclusion, cPLA₂-deficient mice showed decreased allergic symptoms and a loss of bronchial hyperreactivity to methacholine, and female reproductive functions was impaired. These mice are a good model for studying the role of cPLA₂ in physiological and pathological processes. Intensive analysis of the phenotype should enable the side effects to be predicted of cPLA₂ inhibitors that are being developed to treat inflammatory or allergic disorders. □

Methods

Cloning of the cPLA₂ gene and construction of the targeting vector. Murine cPLA₂ genomic DNA was isolated by screening a 129/Sv genomic library in λFixII (Stratagene) with a full-length ORF probe derived from murine cPLA₂ cDNA. Six of 24 isolated clones covered the 137-base exon encoding the lipase consensus motif⁶ and part of the flanking introns. A 4.1-kb PCR product amplified from one of the phage clones using an Expand Hi-Fi kit (Boehringer Mannheim) was used as the upstream homologous region of the targeting vector; the primer set used was 5'-CTCGAGTTCCTATCAGC AGC-3' and 5'-CTCGAGACTCATACTGCGCTTC-3', and 86 bases of the target exon were included in one end of this product. A 4.5-kb *Bam*HI restriction fragment was used as the downstream homologous region; this fragment contains 10 bases derived from the downstream end of the target exon. The targeting vector was constructed in a pBluescript vector (Stratagene), replacing 41 bases of the target exon with a PGK-neo cassette (Fig. 1a).

Transformation of ES cells and selection of targeted cell clones. E14-1 cells were electroporated with *Not*I-linearized targeting vector and *neo*^R or *neo*-resistant cells were selected with G418 (225 µg active weight ml⁻¹) and fluoriodoadenosyluracine (FIAU; 0.2 µM). Three targeted clones were identified after screening 576 doubly-resistant ES clones by Southern blot analysis with the 5' probe after *Eco*RI digestion (Fig. 1b). Additional Southern blot analysis using the 3' probe after *Pst*I digestion and the Neo probe after *Xba*I digestion revealed a lack of rearrangement and of extra insertional events in these clones (data not shown).

Derivation of mutant mice. Two of the three targeted cell lines were injected into C57BL/6J host blastocysts and implanted in pseudopregnant female recipients to generate chimaeric mice. Male mice originating from both cell lines transmitted the 129 genome to their progeny, and were bred to C57BL/6J females. Heterozygous F₁ progeny carrying the disrupted cPLA₂ gene were intercrossed to obtain F₂ mice. Further studies were done on F₂ offspring. The mouse strains used (C57BL/6J and 129/Ola) are congenitally defective in type II PLA₂ activity owing to a frameshift mutation in exon 3 of the type II PLA₂ gene^{25,26}. Type II PLA₂ is a secretory PLA₂ that participates in the release of arachidonate in inflammatory lesions^{3,27}.

Genotype analysis by PCR. Genotypes of mice were determined by PCR using 50 to 100 ng of genomic DNA obtained from tail biopsies as template. The nucleotide sequence of the primers were 5'-CTCTGGTGTGATGAAGGCAC TGTATGAGTC-3' (Fcm), 5'-TCGTGCTTTACGGTATCGCGCTCCCGATT-3' (Neo), and 5'-CCCTACCTACAATGTTACCCAAACTAGC-3' (Rwt); the primer set of Fcm and Neo amplified a 582-bp fragment specific for the *neo* allele, and the set of Fcm and Rwt yielded a 317-bp fragment specific for the wild-type allele. The reaction was carried out using an Ex Taq kit (from Takara, Kyoto, Japan) with 30 cycles of amplification (20 s at 98 °C; 2 min at 68 °C) or 35 cycles (20 s at 98 °C; 3 min at 68 °C) for the *neo* or wild-type allele, respectively.

Northern blot hybridization. 5 µg spleen poly(A)⁺ RNA was electrophoresed on a denaturing gel and transferred onto a Biotodyne (Pall, East Hills) membrane.

cPLA₂ mRNA was detected with a full-length ORF murine cPLA₂ cDNA as probe.

Phospholipase A₂ activity assay and western blotting. Spleen cytosol fractions were prepared from 3-month-old male mice and PLA₂ activity was measured using 2 µM [¹⁴C]arachidonoyl-phosphatidylcholine as substrate; the assay buffer contained 100 mM Tris-HCl, pH 9.0, 4 mM CaCl₂, 1 mg ml⁻¹ BSA²⁸. Protein concentration was determined by Bradford's method (Biorad) using BSA as standard. 50 µg protein was separated on a 7.5% SDS-PAGE and transferred to a polyvinylidene-difluoride (PVDF) membrane (Immobilon, Millipore). cPLA₂ protein was detected using an ECL system (Amersham) and an anti-cPLA₂ monoclonal antibody (Santa Cruz) and an anti-mouse IgG conjugated with horseradish peroxidase.

Stimulated release of eicosanoids and PAF from peritoneal macrophages. Peritoneal macrophages were collected from mice 4 days after intraperitoneal injection of 2 ml of 10% proteose peptone solution (Difco). Cells were cultured in RPMI 1640 medium supplemented with 2% fetal calf serum. The medium was aspirated and replaced with medium containing the Ca²⁺ ionophore A23187 (5 µM) or LPS (1 µg ml⁻¹). The medium was collected and PGE₂ and Cys-LT were quantified using enzyme immunoassay (EIA) kits (Amersham). Levels of PAF in samples containing medium and cells were measured using a radioreceptor binding assay²⁹.

Elicitation of systemic anaphylaxis and bronchopulmonary responses. Male mice (10–12 weeks old) were given an intraperitoneal injection of ovalbumin (100 µg; Sigma), aluminium hydroxide gel (1 mg) and pertussis toxin (300 µg; Kaken, Tokyo)²⁰. Eighteen days later, anaesthetized and mechanically ventilated mice were challenged with 500 µg of OVA in a 100-µl bolus delivered through the jugular vein to elicit systemic anaphylaxis. R_L was measured as described^{21,30}. For histological analysis, lungs were removed intact 5 to 7 min after OVA challenge, frozen in liquid nitrogen, and fixed in Carnoy's solution. Sections were stained with haematoxylin and eosin. A saline challenge to OVA-sensitized mice was carried out as a control. After 20 min of OVA or saline challenge, two deep inhalations were performed to standardize volume history. The reactivity to methacholine aerosol was examined essentially as described²¹.

Mating of mice. One male mouse was mated with 1–3 females. The mice were put together in a cage overnight and separated the next morning. The interval between matings was at least one week for females. Female mice were weighed daily and the date of gestation was determined from changes in body weight.

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- Dennis, E. A. The growing phospholipase A₂ superfamily of signal transduction enzymes. *Trends Biochem. Sci.* **22**, 1–2 (1997).
- Prescott, S. M. A thematic series on phospholipases. *J. Biol. Chem.* **272**, 15043 (1997).
- Murakami, M., Nakatani, Y., Atsumi, G.-I., Inoue, K. & Kudo, I. Regulatory functions of phospholipase A₂. *Crit. Rev. Immunol.* **17**, 225–283 (1997).
- Leslie, C. C. Properties and regulation of cytosolic phospholipase A₂. *J. Biol. Chem.* **272**, 16709–16712 (1997).
- Clark, J. D. *et al.* A novel arachidonic acid-selective cytosolic PLA₂ contains a Ca²⁺-dependent translocation domain with homology to PKC and GAP. *Cell* **65**, 1043–1051 (1991).
- Sharp, J. D. *et al.* Molecular cloning and expression of human Ca²⁺-sensitive cytosolic phospholipase A₂. *J. Biol. Chem.* **266**, 14850–14853 (1991).
- Lin, L. L. *et al.* cPLA₂ is phosphorylated and activated by MAP kinase. *Cell* **72**, 269–278 (1993).
- Kramer, R. M. *et al.* p38 mitogen-activated protein kinase phosphorylates cytosolic phospholipase A₂ (cPLA₂) in thrombin-stimulated platelets. *J. Biol. Chem.* **271**, 27723–27729 (1996).
- Chen, X. S., Sheller, J. R., Johnson, E. N. & Funk, C. D. Role of leukotrienes revealed by targeted disruption of the 5-lipoxygenase gene. *Nature* **372**, 179–182 (1994).
- Goulet, J. L., Snouwaert, J. N., Latour, A. M., Coffman, T. M. & Koller, B. H. Altered inflammatory responses in leukotriene-deficient mice. *Proc. Natl Acad. Sci. USA* **91**, 12852–12856 (1994).
- Irvin, C. G., Tu, Y.-P., Sheller, J. R. & Funk, C. D. 5-Lipoxygenase products are necessary for ovalbumin-induced airway responsiveness in mice. *Am. J. Physiol.* **272**, L1053–L1058 (1997).
- Langenbach, R. *et al.* Prostaglandin synthase 1 gene disruption in mice reduces arachidonic acid-induced inflammation and indomethacin-induced gastric ulceration. *Cell* **83**, 483–492 (1995).
- Dinchuk, J. E. *et al.* Renal abnormalities and an altered inflammatory response in mice lacking cyclooxygenase II. *Nature* **378**, 406–409 (1995).
- Morham, S. G. *et al.* Prostaglandin synthase 2 gene disruption causes severe renal pathology in the mouse. *Cell* **83**, 473–482 (1995).
- Sugimoto, Y. *et al.* Failure of parturition in mice lacking the prostaglandin F receptor. *Science* **277**, 681–683 (1997).
- Sharp, J. D. *et al.* Serine 228 is essential for catalytic activities of 85-kDa cytosolic phospholipase A₂. *J. Biol. Chem.* **269**, 23250–23254 (1994).
- Currie, S., Roberts, E. F., Spaethe, S. M., Roehm, N. W. & Kramer, R. M. Phosphorylation and activation of Ca²⁺-sensitive cytosolic phospholipase A₂ in MCII mast cells mediated by high-affinity Fc receptor for IgE. *Biochem. J.* **304**, 923–928 (1994).
- Nakatani, Y., Murakami, M., Kudo, I. & Inoue, K. Dual regulation of cytosolic phospholipase A₂ in mast cells after cross-linking of Fc epsilon-receptor. *J. Immunol.* **153**, 796–803 (1994).
- Glover, S. *et al.* Translocation of the 85-kDa phospholipase A₂ from cytosol to the nuclear envelope in rat basophilic leukemia cells stimulated with calcium ionophore or IgE/antigen. *J. Biol. Chem.* **270**, 15359–15367 (1995).
- Oettgen, H. C. *et al.* Active anaphylaxis in IgE-deficient mice. *Nature* **370**, 367–370 (1994).

21. Ishii, S. *et al.* Bronchial hyperreactivity, increased endotoxin lethality and melanocytic tumorigenesis in transgenic mice overexpressing platelet-activating factor receptor. *EMBO J.* **16**, 133–142 (1997).
22. Goldberg, V. J. & Ramwell, P. W. Role of prostaglandins in reproduction. *Physiol. Rev.* **55**, 325–351 (1975).
23. O'Neill, C. *et al.* Supplementation of *in-vitro* fertilisation culture medium with platelet activating factor. *Lancet* **2**, 769–772 (1989).
24. Dudley, D. J., Branch, D. W., Edwin, S. S. & Mitchell, M. D. Induction of preterm birth in mice by RU486. *Biol. Reprod.* **55**, 992–995 (1996).
25. MacPhee, M. *et al.* The secretory phospholipase A2 gene is a candidate for the *Mom1* locus, a major modifier of *Apc^{Min}*-induced intestinal neoplasia. *Cell* **81**, 957–966 (1995).
26. Kennedy, B. P. *et al.* A natural disruption of the secretory group II phospholipase A2 gene in inbred mouse strains. *J. Biol. Chem.* **270**, 22378–22385 (1995).
27. Bingham, C. O. III *et al.* A heparin-sensitive phospholipase A2 and prostaglandin endoperoxide synthase-2 are functionally linked in the delayed phase of prostaglandin D2 generation in mouse bone marrow-derived mast cells. *J. Biol. Chem.* **271**, 25936–25944 (1996).
28. Takayama, K. *et al.* Purification and characterization of human platelet phospholipase A2 which preferentially hydrolyzes an arachidonoyl residue. *FEBS Lett.* **282**, 326–330 (1991).
29. Aoki, Y. *et al.* A radioreceptor binding assay for platelet-activating factor (PAF) using membranes from CHO cells expressing human PAF receptor. *J. Immunol. Meth.* **186**, 225–231 (1995).
30. Nagase, T. *et al.* Intercellular adhesion molecule-1 mediates acid aspiration-induced lung injury. *Am. J. Respir. Crit. Care Med.* **154**, 504–510 (1996).

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Reduced fertility and postischaemic brain injury in mice deficient in cytosolic phospholipase A₂

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Phospholipase A₂ (PLA₂) enzymes are critical regulators of prostaglandin and leukotriene synthesis and can directly modify the composition of cellular membranes^{1,2}. PLA₂ enzymes release fatty acids and lysophospholipids, including the precursor of platelet-activating factor, PAF, from phospholipids. Free fatty acids, eicosanoids, lysophospholipids and PAF are potent regulators of inflammation^{1,3,4}, reproduction^{5–7} and neurotoxicity^{1,8,9}. The physiological roles of the various forms of PLA₂ are not well defined. The cytosolic form, cPLA₂, preferentially releases arachidonic acid from phospholipids and is regulated by changes in intracellular calcium concentration^{10,11}. We have now created 'knockout' (cPLA₂^{-/-}) mice that lack this enzyme, in order to evaluate its physiological importance. We find that cPLA₂^{-/-} mice develop normally, but that the females produce only small litters in which the pups are usually dead. Stimulated peritoneal macrophages from cPLA₂^{-/-} animals did not produce prostaglandin E₂ or leukotriene B₄ or C₄. After transient middle cerebral artery occlusion, cPLA₂^{-/-} mice had smaller infarcts and developed less brain oedema and fewer neurological deficits. Thus cPLA₂ is important for macrophage production of inflammatory mediators, fertility, and in the pathophysiology of neuronal death after transient focal cerebral ischaemia.

We used gene targeting in mouse embryonic stem (ES) cells to disrupt an exon in the cPLA₂ gene, generating a null allele (Fig. 1a). Mice were genotyped by Southern blotting (Fig. 1b). The absence of cPLA₂ in homozygous cPLA₂^{-/-} mice was confirmed by western blotting (Fig. 1c); also, Ca²⁺-dependent acylhydrolase activity against the *sn*-2 position of 1-stearoyl-2-[1-¹⁴C] arachidonoyl

phosphatidylcholine was absent in kidney cytosol fractions¹². Kidney cytosolic PLA₂ activity was 2.8 ± 0.4 pmol mg⁻¹ ml⁻¹ in cPLA₂^{+/+} littermates. Brain cPLA₂ activity, identified by gel-filtration chromatography of heparin non-binding activity¹², was not detectable in cPLA₂^{-/-} mice (data not shown).

Peritoneal macrophages from cPLA₂^{-/-} animals released much less arachidonate in response to the receptor-independent stimuli phorbol myristate acetate (PMA) and the Ca²⁺ ionophore A23187 (Fig. 2a). Whereas macrophages from cPLA₂^{+/+} mice responded to lipopolysaccharide (LPS) with enhanced prostaglandin E₂ (PGE₂) release, cPLA₂^{-/-} mice macrophages gave no PGE₂ response to LPS. When stimulated with A23187, macrophages from cPLA₂^{+/+} mice produce large amounts of the leukotrienes B₄ (LTB₄) and C₄ (LTC₄), whereas leukotriene production by cPLA₂^{-/-} mice macrophages is at the lower limit of detection (Fig. 2c, d). PAF production is increased in response to A23187 (2 μm) and PMA (100 nM) in peritoneal leukocytes from cPLA₂^{+/+} but not in cells from cPLA₂^{-/-} mice (data not shown). Although the peritoneal macrophage has other forms of PLA₂ (refs 3, 4), our results indicate that cPLA₂ is the primary form responsible for generation of PGE₂, LTB₄, LTC₄ and PAF in response to inflammatory stimuli.

Heterozygous (cPLA₂^{+/-}) mice appeared normal and, when interbred, yielded litters of normal size (Table 1) with a mendelian

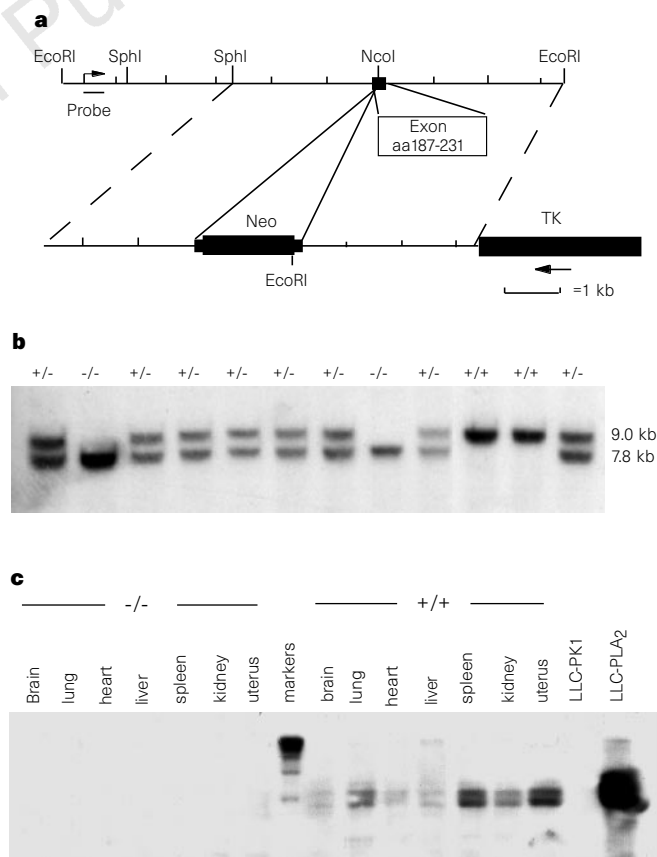


Figure 1 Targeting strategy for inactivation of the cPLA₂ gene and the resulting DNA and protein analysis. **a**, The genomic structure near the exon that was interrupted. Only relevant restriction sites are designated. The interrupted exon began with the codon for amino acid (aa) 187 and ended at the second nucleotide of the codon encoding amino acid 232. Southern blot analysis was performed with the indicated 5' probe. *EcoRI* digestion results in a 9-kb fragment in wild-type DNA and a 7.8-kb fragment in homologous recombinations. **b**, Southern blot analysis of *EcoRI*-digested DNA. **c**, Western blot analysis, using a specific anti-cPLA₂ antibody, of various organs from wild-type and cPLA₂^{-/-} mice. Cell lysates from LLC-PK₁ cells and cPLA₂-overexpressing cells (LLC-PLA₂) provide negative and positive controls. Each lane was loaded with 50 μg total protein.

Table 1 Reproductive data from female heterozygotic and homozygotic cPLA₂ knockout mice

Matings M × F	No. of females mated	No. of females having offspring	No. of litters	Mean litter size	No. of births	No. of dead offspring (% of births)	No. of live offspring
+/- × +/-	15	15	39	7.4	289	13 (4.5%)	276
-/- × +/-	14	14	31	6.9	214	10 (4.7%)	204
-/- × -/-	20	8	17	3.2	54	47 (87%)	7
+/- × -/-	9	4	4	2.8	11	7 (64%)	4
+/+ × -/-	7	6	10	2.9	29	23 (79%)	6

genetic distribution (with wild type/heterozygous/homozygous ratios of: males, 74:130:56; females, 72:128:55). cPLA₂^{-/-} mice develop normally, gain weight at a rate equal to that of wild-type animals and have lifespans of more than 1 year. Female cPLA₂^{-/-} mice, however, become pregnant less frequently and produce small litters that most commonly result in dead pups (Table 1). The dead offspring were larger (1.6 ± 0.1 g; $n = 10$) than pups born from cPLA₂^{+/+} mice (1.2 ± 0.1 g; $n = 10$, $P < 0.005$) and appeared to be anatomically normal, with no gross organ abnormality on post-mortem examination. Removal of offspring at day 18 of pregnancy from a cPLA₂^{-/-} mother yielded four normal viable pups. Almost all of the pups born to homozygous mice null for the prostaglandin synthase 1 (*Ptgs 1*) gene were also born dead. In contrast to our findings, however, *Ptgs1*-mutant females produce normal size litters. Male cPLA₂^{-/-} mice had normal fertility when crossed with wild-type or heterozygous females (Table 1). There was, however, a significant decrease in the reproductive efficiency of male heterozygous and -/- mice when bred to -/- females (Table 1; $P < 0.05$). To determine why pregnancy failed in the (-/-) × (-/-) matings, we examined the formation of vaginal plugs as an indicator of copulation. In -/- females that failed to deliver after mating we found that the initial plug formation was followed several days later by formation of another vaginal plug. The shortest interval between plugs was 7 days. This indicates that pregnancy in -/- females impregnated by -/- males often fails near the time of implantation (~day 4.5), during the period when decidualization occurs in

normal murine pregnancy. As there is usually regulated expression of prostaglandin H synthase in early embryos¹³, the pregnancies may fail as a result of the deficiency or absence of a fetal factor that is elaborated through a cPLA₂-dependent pathway.

The decreased number of pups born to -/- mothers must be due to maternal problems affecting ovulation, oocyte transport or implantation (litter size is the same for +/+, +/- and -/- male matings with -/- females). cPLA₂ is present in uterine (Fig. 1c) and ovarian tissue¹⁴ and prostaglandins are implicated in increasing the vascular permeability of the endometrium during implantation and the induction of decidualization⁵. Uterine PLA₂ activity and levels of PGE₂ and PGF_{2α} are increased during ova implantation¹⁵.

The large size of the pups, which were otherwise normal in appearance, of the cPLA₂^{-/-} female mouse, indicated that there was an additional pathological process operating during delivery. Prostaglandins may regulate uterine contractions and cervical dilatation during labour⁶ and PGF_{2α} is known to be essential for triggering labour⁷. Although other forms of PLA₂ have been identified in the uterus, including in the cervix^{16,17}, the small litters of dead offspring of cPLA₂^{-/-} female mice indicate that other PLA₂ enzymes cannot rescue the fertility defects associated with cPLA₂ deficiency.

There was no significant difference in mean arterial pressure between cPLA₂^{+/+} and cPLA₂^{-/-} siblings, nor in baseline arterial pH, pCO₂, pO₂ or rectal temperature between the two groups. cPLA₂ activity is increased in the brain after ischaemic injury^{10,18}. When we occluded the middle cerebral artery (MCA) with an intraluminal

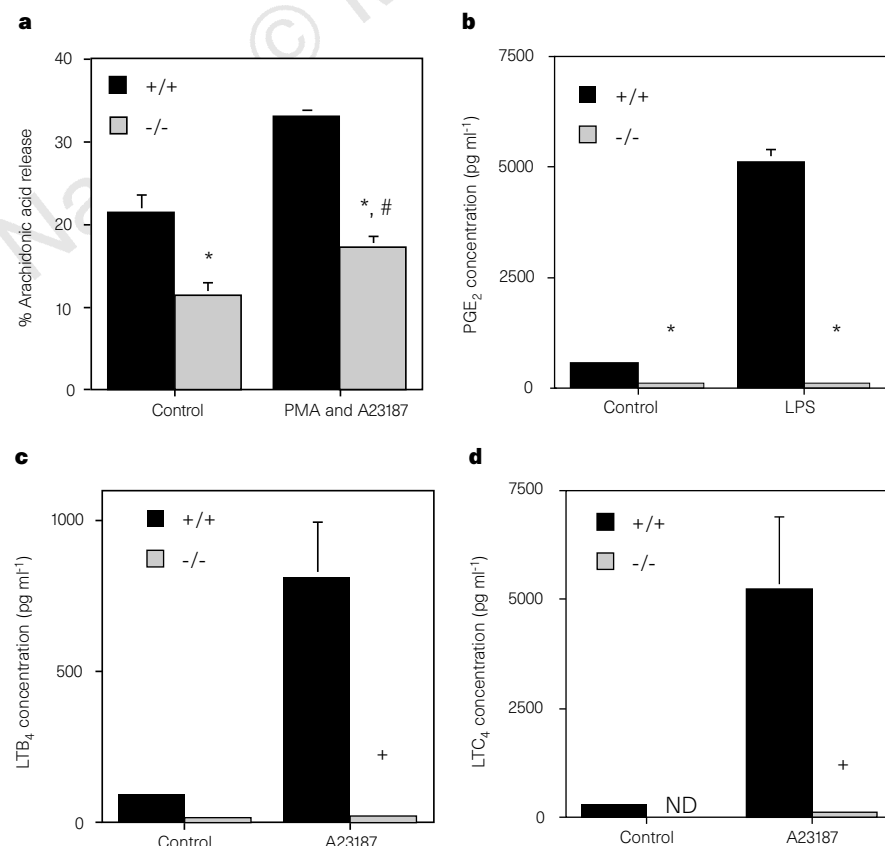


Figure 2 Metabolic and synthetic function is altered in peritoneal macrophages of -/- mice. **a**, Arachidonic acid release, expressed as a percentage of the total label incorporated, is measured from cells prelabeled with [³H]arachidonic acid and exposed to vehicle or 100 nM PMA and 2 μM A23187 for 30 min; $n = 6-10$. **b**, PGE₂ concentration in serum-free RPMI 1640 culture medium of untreated cells or cells incubated with 1,000 ng ml⁻¹ LPS for 6 h; $n = 6$. **c**, LTB₄, and **d**, LTC₄ concentrations in serum-free modified Eagle's medium after 15 min exposure of macrophages to vehicle or 2 μM A23187, $n = 3$. +, $P < 0.05$ and * $P < 0.005$ compared to +/-; # $P < 0.05$ compared to control condition. In this and subsequent figures data are presented as the mean ± s.e.m. ND, not detectable.

Table 2 Reduced neurological deficit in cPLA₂^{-/-} mice after transient focal ischaemia

Group	n	Neurological deficit score				Mean ± s.e.m.
		0	1	2	3	
+/+	12	1	4	7	0	1.50 ± 0.19
-/-	12	5	6	1	0	0.67 ± 0.18*

Neurological deficits were evaluated following 2 h of ischaemia and 22 h of reperfusion and graded from none (0) to most severe (3) as follows: 0, no observable deficit (normal); 1, failure to extend right forepaw upon lifting by the tail (mild); 2, circling to the contralateral side (moderate); 3, leaning to the contralateral side at rest or no spontaneous motor activity (severe). **P* < 0.05, compared to +/+ mice.

filament, both cPLA₂^{+/+} and cPLA₂^{-/-} animals had the same degree of reduction in cerebral blood flow (CBF; to ~20% of baseline), which was sustained for 2 h during ischaemia. After removal of the intraluminal filament, CBF returned to baseline within 30 min. No differences were found in mean arterial blood pressure, or in arterial pO₂, pCO₂, or pH, between wild-type and knockout mice during ischaemia and reperfusion (data not shown). cPLA₂^{-/-} mice had a 34% reduction in infarct volumes (41 ± 7 mm³; *n* = 12) compared to wild-type animals (62 ± 6 mm³; *n* = 12, *P* < 0.05) after 22 h of reperfusion following ischaemia (Fig. 3a). This infarct reduction was most obvious in posterior brain slices (Fig. 3b). Wild-type mice developed significant brain oedema ipsilateral to the MCA occlusion, with an increase of 4.0 ± 0.8% in brain water compared to the contralateral side (*P* < 0.05; *n* = 7 in each group). By contrast, brain water in the hemisphere ipsilateral to MCA occlusion did not increase significantly in cPLA₂^{-/-} mice. When the effects of oedema on the apparent infarction size are eliminated, the relative amount of protection in cPLA₂^{-/-} mice (37%) remains about the same (cPLA₂^{-/-}: 16.4 ± 1.8%; cPLA₂^{+/+}: 26.1 ± 2.5% of hemisphere volume; *n* = 12, *P* < 0.05). The neuroprotective effect was also seen in brains examined three days after the transient ischaemic episode, when infarct volumes were 92 ± 9 mm³ in cPLA₂^{+/+} mice, and 66 ± 7 mm³ in cPLA₂^{-/-} mice, respectively (*P* < 0.05, *n* = 8 in each group). There were no systematic differences in vascular anatomy of the brain between cPLA₂^{+/+} and cPLA₂^{-/-} animals, as determined by microscopic examination of the vasculature injected with india ink. Consistent with the degree of infarct reduction, fewer functional neurological deficits^{19,20} were observed in cPLA₂^{-/-} mice when compared with cPLA₂^{+/+} animals 22 h after ischaemia (Table 2).

The finding that cPLA₂^{-/-} mice are partially protected after cerebral ischaemia and reperfusion indicates that cPLA₂ may be

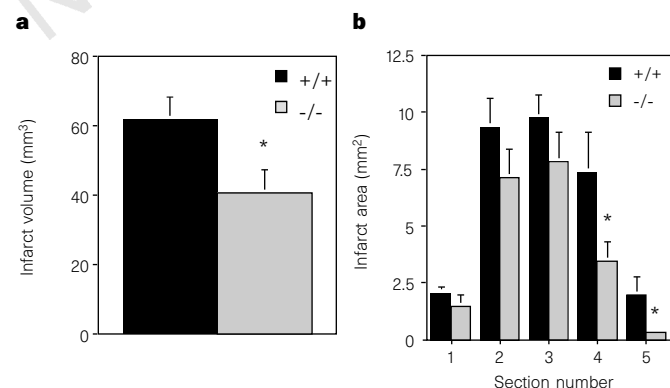


Figure 3 Middle cerebral artery (MCA) occlusion results in smaller infarcts in knockout mice. **a**, Transient MCA occlusion produces smaller infarct volumes in cPLA₂^{-/-} mice compared to wild-type animals. Twenty-two hours after transient MCA occlusion for 2 h, brains were coronally sectioned into five 2-mm-thick slices from rostral to caudal (sections 1–5). Total infarct volume was calculated by summation of TTC-stained infarction volume from all sections. **P* < 0.05 compared to wild type; *n* = 12 in each group. **b**, The reduction in infarct size is more pronounced in the posterior coronal sections of the knockout mice. **P* < 0.05.

important in the pathogenesis of reperfusion injury in the brain. cPLA₂ may contribute to brain injury by a direct effect on cellular membranes, or indirectly through generation of inflammatory and vasoconstrictive mediators^{9,10,21–23}. Whereas other PLA₂ enzymes and various lipases have been implicated in brain arachidonic acid release and eicosanoid production^{1,10,24}, the functional significance of an absence of cPLA₂ attests to the importance of this form of the enzyme as a mediator of brain injury.

In conclusion, our results demonstrate the importance of cPLA₂ in the generation of bioactive lipids, in fertility and reproduction, and in postischaemic brain injury. Our findings link arachidonic acid generated by cPLA₂ to the production of cyclooxygenase and lipoxygenase products and of PAF in inflammatory cells. This link has implications for the regulation of eicosanoid and PAF production in the process of inflammation and reperfusion injury, as well as for the normal control of fertility and parturition. □

Methods

Targeted inactivation of the cPLA₂ gene. A 129/Sv mouse genomic library was screened by hybridization with a 900-bp 5' *SalI*/*EcoRV* fragment of human cPLA₂ (ref. 11). A 9-kb *EcoRI* fragment containing one 137-bp exon, beginning with the complete codon for amino acid 187 and ending with the second nucleotide of the codon encoding amino-acid 232 of the mouse protein sequence¹¹, was cloned into pBluescript. A PGK-neo-poly(A) cassette containing an *EcoRI* site at its 5' end was inserted into an *NcoI* site within the exon in a 3'–5' orientation and the PGK-thymidine kinase gene was inserted into the *XbaI* site 3.5 kb downstream from the exon. A 400-bp intronic fragment ~5 kb upstream from the exon was PCR-amplified for use as a probe. The resulting targeting vector contained 5' and 3' homology regions of ~2.5 and 3.5 kb respectively. The vector was electroporated into J1 ES cells and neomycin-resistant clones were isolated. Germline transmission was achieved with two clones, resulting in mouse strains with identical reproductive phenotypes.

Western blots and PLA₂ activity. Mouse organs were Dounce-homogenized and the cytosolic protein fraction was recovered as the 100,000g supernatant. Immunodetection of cPLA₂ was performed on cytosolic fractions, as described¹², using a polyclonal antibody raised against the first 129 amino acid residues of cPLA₂. Cytosolic protein fractions derived from wild-type and cPLA₂^{-/-} mice were assayed for cPLA₂ activity as described²⁵.

Peritoneal macrophage arachidonic acid, eicosanoid and PAF production. Resident peritoneal macrophages were collected by lavage²⁶ and seeded at 1–2 × 10⁶ cells ml⁻¹ on tissue-culture plates in RPMI 1640 medium for 18 h. For PAF analysis, peritoneal leukocytes were collected 36 h after intraperitoneal injection of 2% casein. Arachidonic acid release was determined²⁵ after labelling cells for 2 h with [³H]arachidonic acid (0.2 μCi ml⁻¹). PGE₂ and leukotriene concentrations in the culture medium were measured using radioimmunoassay (Sigma) or ELISA (Cayman Chemical), respectively. PAF was assayed by standard techniques²⁷.

Physiological parameters and cerebral blood flow. Mice weighing 20 to 25 g were anaesthetized with 1% halothane in 70/30% nitrous oxide/oxygen by mask, and the rectal temperature was maintained between 36.5–37.5 °C. Blood pressure was monitored and arterial blood samples were analysed at 37 °C for pH, pCO₂ and pO₂ 10 min before MCA occlusion, 1 h into ischaemia and 30 min after reperfusion. An equal amount of saline was injected after every sampling. Regional CBF was measured by laser-Doppler flowmetry with a fibre-optic probe placed 2 mm posterior, 6 mm lateral to the bregma on the ipsilateral hemisphere, the site supplied by the proximal segment of the MCA¹⁹.

Production of focal cerebral ischaemia and measurement of brain infarction and oedema. Transient focal cerebral ischaemia was induced by occlusion of the MCA using an intraluminal filament technique¹⁹. The left common and external carotid arteries were ligated and microvascular clips were placed on the internal carotid and pterygopalatine arteries. A silicone-coated nylon monofilament was introduced through the external carotid artery into the internal carotid artery and advanced to occlude the MCA. Two hours later, the filament was removed and the common carotid artery reopened.

Twenty-two or 72 hours after reperfusion, brains were removed, sectioned coronally into five 2-mm slices, and the infarct area was measured using 2% 2,3,5-triphenyltetrazolium chloride (TTC) (22 h) or haematoxylin and eosin

(72 h) staining. The infarction volume was calculated by summing the infarct volumes of sections. Infarct size (%) was also calculated by using the following formula: (contralateral volume – ipsilateral undamaged volume) $\times$ 100/contralateral volume to eliminate effects of oedema¹⁹.

Brain oedema was evaluated in dissected ipsilateral (ischaemic) and contralateral (non-ischaemic) hemispheres. The fresh samples were weighed and dried. Water content (%) was calculated as: $100 \times (\text{wet weight} - \text{dry weight}) / \text{wet weight}$ (ref. 28).

Statistical analysis. Reproductive genetics were evaluated by χ^2 testing and mating efficiency of $+/+$ and $-/-$ males to $-/-$ females was compared with the one-tailed Fisher's test. The Mann–Whitney *U*-test was used to compare neurological deficit scores. Other data were analysed by Student's *t*-test or analysis of variance (ANOVA) followed with the Bonferroni test. Statistical significance was assigned to comparisons with $P < 0.05$. Data are expressed as mean $\pm$ s.e.m.

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1. Bonventre, J. V. Phospholipase A₂ and signal transduction. *J. Am. Soc. Nephrol.* **3**, 128–150 (1992).
2. Malis, C. D. & Bonventre, J. V. Mechanism of calcium potentiation of oxygen free radical injury to renal mitochondria. A model for post-ischemic and toxic mitochondrial damage. *J. Biol. Chem.* **261**, 14201–14208 (1986).
3. Dennis, E. A. The growing phospholipase A₂ superfamily of signal transduction enzymes. *Trends Biochem. Sci.* **22**, 1–2 (1997).
4. Teslenko, V., Rogers, M. & Lefkowitz, J. B. Macrophage arachidonate release via both the cytosolic Ca²⁺-dependent and -independent phospholipases is necessary for cell spreading. *Biochim. Biophys. Acta* **1344**, 189–199 (1997).
5. Abrahamsohn, P. A. & Zorn, T. M. T. Implantation and decidualization in rodents. *J. Exp. Zool.* **266**, 603–628 (1993).
6. Challis, J. R. G. & Lye, S. J. in *The Physiology of Reproduction* (eds Knobil, E. & Neill, J. D.) 985–1031 (Raven, New York, 1994).
7. Sugimoto, Y. *et al.* Failure of parturition in mice lacking the prostaglandin F receptor. *Science* **277**, 681–683 (1997).
8. Gilboe, D. D. *et al.* Recovery of postischemic brain metabolism and function following treatment with a free radical scavenger and platelet-activating factor antagonists. *J. Neurochem.* **56**, 311–319 (1991).
9. Bonventre, J. V. & Koroshetz, W. J. Phospholipase A₂ (PLA₂) activity in gerbil brain: Characterization of cytosolic and membrane-associated forms and effects of ischemia and reperfusion on enzymatic activity. *J. Lipid Med.* **6**, 457–471 (1993).
10. Rordorf, G., Uemura, Y. & Bonventre, J. V. Characterization of phospholipase A₂ (PLA₂) activity in gerbil brain. Enhanced activities of cytosolic, mitochondrial and microsomal forms after ischemia and reperfusion. *J. Neurosci.* **11**, 1829–1836 (1991).
11. Clark, J. D. *et al.* A novel arachidonic acid-selective cytosolic PLA₂ contains a Ca⁺⁺ dependent translocation domain with homology to PKC and GAP. *Cell* **65**, 1043–1051 (1991).
12. Kim, D. K. & Bonventre, J. V. Purification of a 100 kDa phospholipase A₂ from spleen, lung and kidney. Antiserum raised to pig spleen phospholipase A₂ recognizes a similar form in bovine lung, kidney and platelets, and immunoprecipitates phospholipase A₂ activity. *Biochem. J.* **294**, 261–270 (1993).
13. van der Weiden, R. M. F., Wisse, L. J., Helmerhorst, F. M., Keirse, M. J. N. C. & Poelmann, R. E. Immunohistochemical and ultrastructural localization of prostaglandin H synthase in the preimplantation mouse embryo. *J. Reprod. Fert.* **107**, 161–166 (1996).
14. Kol, S., Ben-Shlomo, L., Ando, M., Payne, D. W. & Adashi, E. Y. Interleukin-1 beta stimulates ovarian phospholipase A₂ (PLA₂) expression and activity: up-regulation of both secretory and cytosolic PLA₂. *Endocrinology* **138**, 314–321 (1997).
15. Novaro, V. *et al.* Interaction between uterine PGE and PGF_{2α} production and the nitridergic system during embryonic implantation in the rat. *Prostaglandins* **51**, 363–376 (1996).
16. Rice, G. E. Secretory type II phospholipase A₂ and the generation of intrauterine signals. *Repro. Fert. Dev.* **7**, 1471–1479 (1995).
17. Rajabi, M. R. & Cybulsky, A. V. Phospholipase A₂ activity is increased in guinea pig uterine cervix in late pregnancy and at parturition. *Am. J. Physiol.* **269**, E940–E947 (1995).
18. Clemons, J. A. *et al.* Reactive glia express cytosolic phospholipase A₂ after transient global forebrain ischemia in the rat. *Stroke* **27**, 527–535 (1996).
19. Huang, Z. *et al.* Effects of cerebral ischemia in mice deficient in neuronal nitric oxide synthase. *Science* **265**, 1883–1885 (1994).
20. Yang, G. *et al.* Human copper-zinc superoxide dismutase transgenic mice are highly resistant to reperfusion injury after focal cerebral ischemia. *Stroke* **25**, 165–170 (1994).
21. Bazan, N. G. Arachidonic acid in the modulation of excitable membrane function and the onset of brain damage. *Ann. NY Acad. Sci.* **559**, 1–16 (1989).
22. Moskowitz, M. A., Kiwak, K. J., Hekimian, K. & Levine, L. Synthesis of compounds with properties of leukotriene C₄ and D₄ in gerbil brains after ischemia and reperfusion. *Science* **224**, 886–889 (1984).
23. Verity, M. A. Mechanisms of phospholipase A₂ activation and neuronal injury. *Ann. NY Acad. Sci.* **679**, 110–120 (1993).
24. Ross, B. M., Kim, D. K., Bonventre, J. V. & Kish, S. J. Characterization of a novel phospholipase A₂ activity in human brain. *J. Neurochem.* **64**, 2213–2221 (1995).
25. Sapirstein, A., Spech, R. A., Witzgall, R. & Bonventre, J. V. Cytosolic phospholipase A₂ (PLA₂), but not secretory PLA₂, potentiates hydrogen peroxide cytotoxicity in kidney epithelial cells. *J. Biol. Chem.* **271**, 21505–21513 (1996).
26. Ausubel, F. M. *et al.* *Current Protocols in Molecular Biology* (Wiley, New York, 1987).
27. Winkler, J. D., Bolognese, B. J., Roshak, A. K., Sung, C.-M. & Marshall, L. A. Evidence that 85 kDa phospholipase A₂ is not linked to CoA-independent transacylase-mediated production of platelet-activating factor in human monocytes. *Biochim. Biophys. Acta* **1997**, 173–184 (1997).
28. Hara, H., Huang, P. L., Panahian, N., Fishman, M. C. & Moskowitz, M. A. Reduced brain edema and infarction volume in mice lacking the neuronal isoform of nitric oxide synthase after transient MCA occlusion. *J. Cereb. Blood Flow Metab.* **16**, 605–611 (1996).

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Miranda directs Prospero to a daughter cell during *Drosophila* asymmetric divisions

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Asymmetric cell division is a general process used in many developmental contexts to create two differently fated cells from a single progenitor cell. Intrinsic mechanisms like the asymmetric transmission of cell-fate determinants during cell division, and extrinsic cell-interaction mechanisms, can mediate asymmetric divisions^{1–3}. During embryonic development of the *Drosophila* central nervous system, neural stem cells called neuroblasts divide asymmetrically to produce another multipotent neuroblast and a ganglion mother cell (GMC) of more restricted developmental potential. Intrinsic mechanisms promote asymmetric division of neuroblasts: for example, the transcription factor Prospero localizes to the basal cell cortex of mitotic neuroblasts and then segregates exclusively into the GMC, which buds off from the basal side of the neuroblast^{4–6}. In the GMC, Prospero translocates to the nucleus, where it establishes differential gene expression between sibling cells. Here we report the identification of a gene, *miranda*, which encodes a new protein that co-localizes with Prospero in mitotic neuroblasts, tethers Prospero to the basal cortex of mitotic neuroblasts, directing Prospero into the GMC, and releases Prospero from the cell cortex within GMCs. *miranda* thus creates intrinsic differences between sibling cells by mediating the asymmetric segregation of a transcription factor into only one daughter cell during neural stem-cell division.

We previously identified a ~30-amino-acid domain in the Prospero protein, termed the asymmetry domain, that is necessary for the asymmetric segregation of Prospero exclusively into the GMC during neuroblast divisions⁷. On the basis of the assumption that this domain interacts with other proteins that partition Prospero to GMCs, we used the yeast two-hybrid system⁸ to search for proteins that interact specifically with the Prospero asymmetry domain. We identified one such species of clone (Fig. 1a): both the partial and full-length species of these clones interacted with a nearly full-length version of the Prospero protein but not with a nearly identical protein that lacked the Prospero asymmetry domain (Fig. 1a, and see Methods). These data indicate that the protein encoded by these clones physically associates with Prospero through the asymmetry domain. We designate this gene *miranda*.

The full-length complementary DNA of *miranda* encodes an 830-amino-acid protein (Fig. 1b). Although *Miranda* is a new type of protein, it contains several known sequence motifs. The middle portion of the protein is homologous with the region of the myosin rod that forms the coiled-coil structure. Two leucine-zipper motifs are found in the carboxy-terminal half of the protein and eight consensus sites for phosphorylation by protein kinase C (PKC) are located in the C-terminal 100 amino acids (Fig. 1b). Sequence analysis of a *miranda* allele suggests these PKC sites may regulate the ability of *miranda* to bind Prospero (see later).

To follow the expression and subcellular localization of the

Miranda protein during embryogenesis and neuroblast cell division, we raised antibodies against a C-terminal polypeptide of the Miranda protein (see Methods). Before neurogenesis, Miranda is expressed in the ectoderm but not in the mesodermal primordium (data not shown). During neurogenesis, neuroblasts, sensory organ precursor cells of the peripheral nervous system, and midgut stem cells in the endoderm, express Miranda. All of these cells also synthesize and segregate Prospero to one of two daughter cells during their asymmetric divisions^{7,9,10}.

To investigate the relation between Miranda and Prospero protein localization, we followed the subcellular localization of these two proteins during neuroblast cell division (Fig. 2). In interphase, we detected Miranda at low levels in the cytoplasm and throughout the cell cortex (Fig. 2c). During mitosis, Miranda localizes to the basal cortex, and then segregates asymmetrically to sibling GMCs (Fig. 2d–f), as does Prospero^{7,9,10}. The co-localization of Miranda and Prospero in mitotic neuroblasts is consistent with the notion that Miranda associates with Prospero at the cell cortex (Fig. 2a, b). Immediately following cytokinesis, Miranda and Prospero co-localize in the cell cortex of GMCs. This event is fleeting, as Miranda quickly becomes undetectable while Prospero translocates to GMC nuclei (Fig. 2a). Thus, in neuroblasts, and briefly in GMCs, Miranda and Prospero co-localize to the cell cortex, then, concomitant with a decrease in Miranda expression in the GMC, Prospero translocates into the GMC nucleus.

To analyse the function of Miranda *in vivo*, we generated six mutant alleles of *miranda* during a large-scale screen to identify mutations that alter neural fate in the developing embryonic central nervous system (CNS). All six alleles fall into a single non-complementation group. These mutations are within the *miranda* gene because: embryos from three of these alleles fail to express Miranda protein; we sequenced the entire open reading frame (ORF) of two putative *miranda* alleles and found molecular lesions in *miranda* in both alleles (see below); and we rescued the mutant phenotype of one putative *miranda* allele with a *miranda* transgene (see Methods).

To investigate whether Miranda promotes the asymmetric segregation of Prospero into GMCs during neuroblast division, we followed the subcellular dynamics of Prospero localization in the six different *miranda* alleles. Prospero is mislocalized in all six alleles

(Fig. 3). Five alleles show an identical pattern: Prospero localizes throughout the cytoplasm of neuroblasts during mitosis and then partitions equally into both the GMC and the neuroblast following neuroblast division (Fig. 3c, d). Consequently, Prospero accumulates in the nuclei of both neuroblasts and GMCs (Fig. 3c). The ubiquitous expression of the *miranda* transgene in embryos homozygous mutant for *miranda*^{ZZ176} restores the normal subcellular localization and segregation of Prospero (Fig. 3g, h).

One allele, *miranda*^{RR127}, exhibits a Prospero mislocalization phenotype distinct from the other five alleles. In *miranda*^{RR127} embryos, the initial cortical localization of Prospero and its subsequent segregation into GMCs appears essentially normal (Fig. 3f). However, following cytokinesis Prospero fails to translocate to the nucleus and persists at the cell cortex (Fig. 3e). We infer from this observation that the Miranda^{RR127} protein can bind to but cannot release Prospero efficiently in GMCs. Our mutational analysis indicates that Miranda performs two separate functions: first, it tethers Prospero to the basal cortex of neuroblasts, directing Prospero into GMCs which bud off from the basal side of neuroblasts; and second, Miranda, or a regulator of Miranda function, is required to release Prospero from the GMC cell cortex.

In *prospero* mutant embryos, the subcellular localization of Miranda is normal (Fig. 3i). These epistatic experiments, together with our genetic analysis, indicate that Miranda localizes Prospero to the cell cortex in mitotic neuroblasts in order to segregate the transcription factor into GMCs. The Numb protein and Prospero are almost identically localized and show similar segregation dynamics and both promote asymmetric divisions^{11–13}. To test whether *miranda* can mediate the unequal partitioning of Numb, we followed Numb localization in *miranda* mutant embryos. In contrast to Prospero, we detected no change to the asymmetric segregation of Numb in *miranda* mutant embryos (data not shown).

Transcripts from the *miranda*⁸⁸¹⁷⁶ gene contain a 169-base-pair (bp) deletion that causes a frameshift followed immediately by a stop codon. This produces a truncated protein lacking the C-terminal 384 amino acids (Fig. 1b). The region missing in this deletion includes the entire amino-acid sequence encoded by the original clone isolated in the two-hybrid screen. This, together with the delocalization of Prospero in embryos mutant for this *miranda*

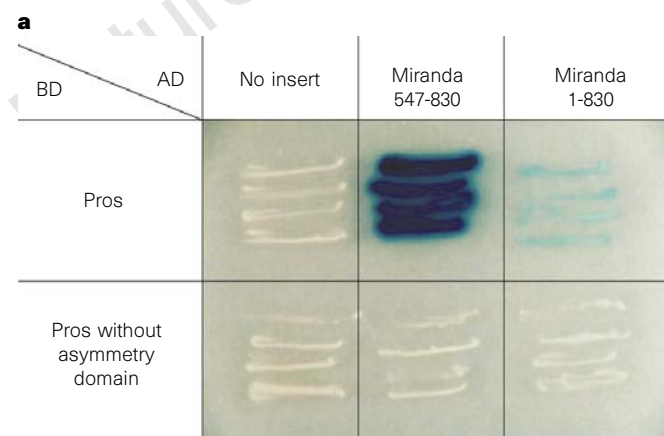


Figure 1 Identification of the *miranda* gene. **a**, Yeast strains expressing a GAL4-binding domain (BD) fused to Pros (upper row) or Pros lacking the asymmetry domain (lower row), with a GAL4 activation domain alone (left column), fused to the initially isolated Miranda sequence (middle column) or full-length Miranda (right column). Blue staining indicates interactions. **b**, Amino-acid sequence of Miranda. Leucine-zipper motifs are underlined. Shaded are Pro-Lys/Arg-Ser/Thr repeats. The arrow indicates the N terminus of the partial cDNA clone isolated by two-hybrid screening. Arrowhead and double arrowhead show the beginning of the missing amino-acid sequence in *miranda*^{ZZ176} and *miranda*^{RR127} mutant proteins, respectively.

b	
MSFSKAKLKRFDVDAICGSPAASNSAGSAGSATPTASSAAAAPPTVQ	50
PERKEQIEKFFKDAVRFASSSKEAKEFAIPKEDKSKGLRLFRTPSLPQR	100
LRFRPTPSHTDTATGSGSGASTAATPLHSAATIPVKEAKSASRLKGKEA	150
LQYEIRHKNELIESQLSQLDLVLRHVDQLKEAFKLRHEHELATSKTDRL	200
TEALTSENLSHKALNEQMGQEHADLLERLAAMEQQLQQQDHEHERQVEAL	250
VAESEALRLANELLQTANEDRQKVVEQLQAQLSALQADVAQAREHCSLEQ	300
AKTAENIELVENLQKTNASLLADVVQLKQIEQDALSYGQEAQSCQAELE	350
CLKVERNILKNDLANKCTLIRSLQDELLDKNCEIDAHCDTIRQLCREQAR	400
HTEQQQAVAKVQQQVESDLESAREKSYWAELEDKQKLAENELIKTEL	450
EKQDVMVLELTINILMRDEKLEKCEBOLRNGIDYITQLSDALQQQLVQ	500
LKQDMAKTIITEKYNYQLTLINIRATVNIILMERLKKSDADVEQYRALESV	550
QLAKGALEQSYLVQLQADABQLRQQLTESQDALNALRSSQTLQSEVSLKE	600
SLHELLAGEAETLAKFNQIANSFOERIDGDAQLAHYHELRKRDRETRAY	650
MVDMKKALDEFATVLOFAQLELUNKEOMLVKRVREECEQLKLENIALKSKQ	700
PGSASLLGTPGKANRSNTDLEKIDEDLDCSELRSDCSEKITITWLLNSSDK	750
CVRQDTTSEINELLSAGKSSPRPAEPTPKAEHTPTSPRTPTHTPTPTPSA	800
ASTPKKTVLFAKENVSPSPQKQVLKARNI	830

allele, suggests the Miranda^{ZZ176} protein lacks a domain necessary to bind Prospero. Transcripts from the *miranda*^{RR127} gene contain a small 10-bp deletion that induces a frameshift replacing the C-terminal 103 amino acids of Miranda with an unrelated stretch of 112 amino acids (Fig. 1b). In *miranda*^{RR127} embryos, Prospero protein localizes normally to the basal cortex of neuroblasts and segregates into GMCs, where it now inappropriately fails to fall off the cortex (Fig. 3e, f). Thus, the C-terminal 103 amino acids are unlikely to act as a tether for Prospero to the cell cortex, but appear to regulate the release of Prospero from the cell cortex of GMCs. The 280 amino acids between residues 445–727 in Miranda may contain a domain necessary for binding Prospero while the C-terminal 100 amino acids may participate in the release of Prospero in GMCs. This region of Miranda contains Pro-Arg/His-Thr/Ser repeats that satisfy the consensus for PKC phosphorylation sites (Fig. 1b), raising the possibility that phosphorylation regulates the interaction of Miranda with Prospero. Alternatively, the C-terminal region may signal for the proteolytic cleavage of Miranda in GMCs, which would then enable Prospero to translocate to the nucleus: this is consistent with the temporal correlation between the disappearance

of Miranda protein and the nuclear translocation of Prospero in GMCs. However, in wild-type or mutant *miranda* embryos that ubiquitously express Miranda (see Methods), Prospero translocates to the nucleus of GMCs even though these cells still contain high levels of Miranda at their cell cortex (arrowheads in Fig. 3g). This suggests that degradation of Miranda is not a prerequisite for nuclear translocation of Prospero and favours our first hypothesis.

The identification of mutations in *miranda* allowed us to analyse the overall effect that lack of *miranda* function has on CNS development. All five *miranda* mutants that cause Prospero to fall off the cortex of neuroblasts exhibit essentially identical CNS phenotypes, as illustrated for *miranda*^{L44} embryos. The axon tracts are grossly defective. Longitudinal connectives are generally either broken or severely reduced in width and commissures are often partially or completely fused (Fig. 4f). The axonal phenotype of *miranda*^{RR127} embryos is similar but much milder than the other alleles (Fig. 4i).

Defects in axon tract formation are often the result of incorrect neuronal determination^{14,15}. We therefore followed the expression of the *even-skipped* (*eve*) gene in embryos mutant for all six *miranda*

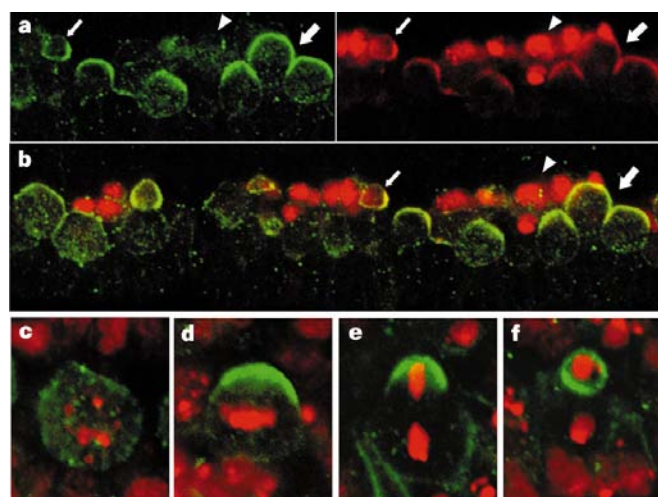


Figure 2 Miranda and Prospero localization in neural stem cells. Apical is at the bottom. **a, b**, Lateral view of the embryonic central nervous system showing Miranda (**a**, left, green) or Prospero localization (**a**, right, red) as well as the merged image showing co-localization of both Miranda and Prospero (**b**). Miranda and Prospero co-localize to the basal cortex of mitotic neuroblasts (thick arrows) and in the cell cortex of GMCs immediately following cytokinesis (thin arrows). Shortly after cytokinesis (arrowhead), Prospero but not Miranda translocates to the nucleus (arrowhead). **c–f**, Cell-cycle-dependent localization of Miranda in neuroblasts. The dynamics of Miranda expression (green) and chromosomal condensation (red) during **c**, interphase; **d**, metaphase; **e**, anaphase; and **f**, immediately after cytokinesis.

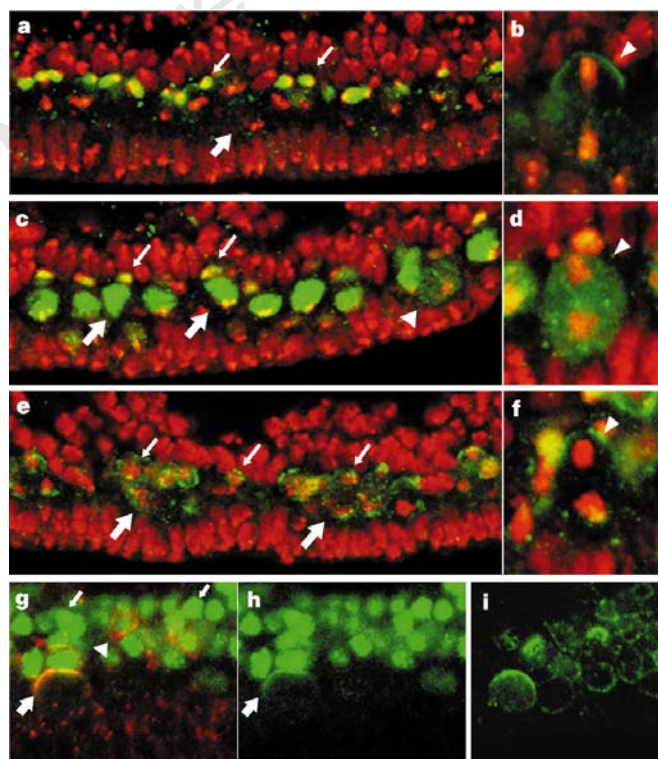


Figure 3 Prospero localization in embryos mutant for *miranda*. Apical is at the bottom, Prospero expression is green, chromosomes are red. **a–f**, GMCs (thin arrows) and interphase neuroblasts (thick arrows) in wild-type (**a, b**), *miranda*^{ZZ176} (**c, d**) and *miranda*^{RR127} (**e, f**) embryos. Arrowhead in **c** indicates a mitotic neuroblast; in **b, d** and **f**, arrowheads highlight an anaphase neuroblast in wild-type (**b**), *miranda*^{ZZ176} (**d**), and *miranda*^{RR127} (**f**) embryos. **g, h**, Ubiquitous expression of a *miranda* transgene (red in **g**) in homozygous *miranda*^{ZZ176} embryo rescues the Prospero delocalization phenotype: Prospero (green in **g, h**) localizes to the cortex of mitotic neuroblasts (thick arrows, **g, h**), segregates into the GMC, and translocates into the GMC's nucleus (thin arrows, **g**). Arrowhead in **g** indicates a GMC that contains nuclear Prospero but maintains Miranda at the cortex. **i**, Miranda (green) is normally localized in *pros*^{C7} embryos⁶.

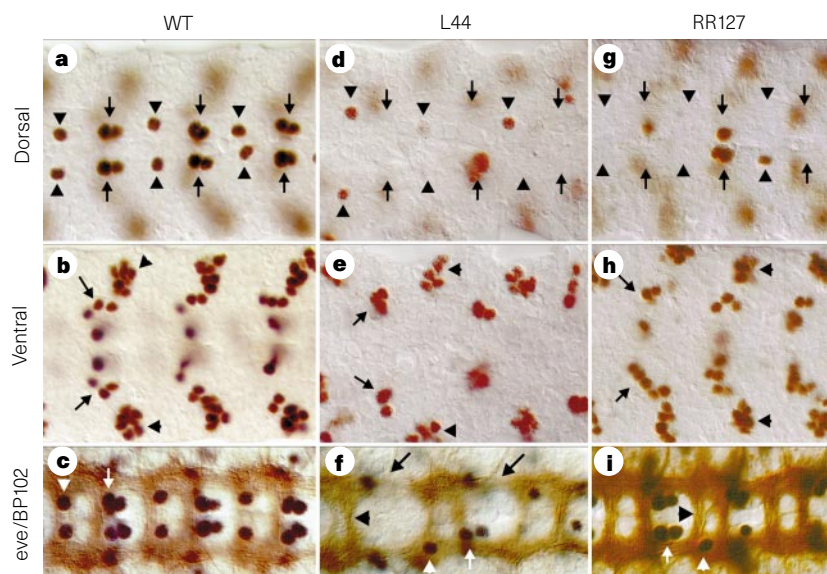


Figure 4 Mutations in *miranda* disrupt both the neuronal and the axonal scaffold. Dorsal (**a**, **c**, **d**, **f**, **g**, **i**) and ventral (**b**, **e**, **h**) views of three consecutive segments of wild-type (WT; **a**–**c**), *miranda*^{L44} (**d**–**f**) and *miranda*^{RR127} (**g**–**i**) mutant embryos labelled for Eve or Eve and the axonal marker BP102 (**c**, **f**, **i**). Anterior is left. Arrows in **a**, **d**, **g**, and white arrows in **c**, **f**, **i**, indicate where aCC/pCC normally form. Arrowheads in **a**, **d**, **g** and white arrowheads in **c**, **f**, **i** mark where RP2 normally forms. In **b**, **e**, **h** arrows mark the U/CQ neuron cluster and arrowheads the EL neuron cluster. In wild-type embryos, RP2 and aCC/pCC occupy stereotyped positions along the axonal scaffold in the dorsal region of the nerve cord

(**a**, **c**), whereas the U/CQ and EL neurons reside in stereotyped locations ventrally (**b**). In *miranda*^{L44} embryos, both RP2 and aCC/pCC form between 30–50% of the time (**d**, **f**) and the number of U/CQ and EL neurons is reduced by roughly one-half. *miranda*^{L44} embryos also exhibit broken and thin longitudinal connectives (arrows, **f**) and the partial fusion of the anterior and posterior commissures (arrowhead, **f**). In *miranda*^{RR127} embryos, RP2 forms only 10% of the time whereas aCC/pCC form 40% of the time (**g**, **i**); however, development of U/CQ and EL neurons is essentially wild-type (**h**). Also, although the axon tracts appear close to wild-type, the longitudinal connectives are thin and the commissures do not fully separate (**i**).

alleles. A stereotyped pattern of GMCs and neurons express *eve*¹⁵. The well characterized aCC/pCC, RP2, CQ, U, and EL neurons all express *eve*^{16,17} (Fig. 4a–c). In *prospero* mutant embryos, the aCC/pCC and RP2 neurons fail to express *eve* and most U and CQ neurons also fail to express *eve*⁴. We expected all *miranda* alleles to show reduced *prospero* activity in the GMC either because Prospero inappropriately segregates into both neuroblasts and GMCs, or because Prospero fails to translocate efficiently into the nuclei of GMCs (*miranda*^{RR127}). This predicts that the Eve CNS phenotype of *miranda* mutant embryos might resemble the Eve CNS phenotype of *prospero* mutant embryos if *miranda* exerts its effect through its ability to bind, segregate and release Prospero. This is the case for both categories of *miranda* mutants. In the five alleles in which Prospero falls off the cortex of neuroblasts, we observed a reduction by about one-half in the number of RP2, aCC/pCC, U and CQ neurons that express *eve* (Fig. 4d–f). Consistent with a decrease in the level of Prospero protein distributed into GMCs, this phenotype resembles a weak *prospero* phenotype. In addition, we observed a one-half reduction in the number of *eve*-expressing EL neurons; in *prospero* mutant embryos, all EL neurons form normally. This additional *eve* phenotype may result from the ectopic expression of Prospero in neuroblasts or from defects in the partition of other factors dependent upon Miranda function; the construction and analysis of fly lines doubly mutant for *miranda* and *prospero* should clarify this.

miranda^{RR127} embryos exhibit a similar but distinct phenotype: an *eve*-expressing RP2 forms only 12% of the time ($n = 202$); aCC/pCC form 38% of the time ($n = 206$); U, CQ and EL neuron development is essentially as wild-type (Fig. 4g–i). In moderate *prospero* mutant embryos no, or very few, *eve*-positive RP2 or aCC/pCC neurons develop but some *eve*-positive U and CQ neurons do. This suggests that there is a more stringent requirement for *prospero* in RP2 and aCC/pCC than the U and CQ neurons. The *eve* phenotype in *miranda*^{RR127} is consistent with this, because RP2 and aCC/pCC are preferentially affected in this mutant background

in which the nuclear translocation of *prospero* is blocked or greatly delayed.

We have shown that the *miranda* gene encodes a protein that is required to anchor the transcription factor Prospero to one side of the cell cortex so that Prospero is asymmetrically distributed during cell divisions to one daughter cell. Our phenotypic and sequence analyses of different *miranda* mutations reveal two separable functions performed by Miranda during asymmetric division of neuroblasts, in which Miranda tethers Prospero to the basal cell cortex of neuroblasts during mitosis and also regulates the release of Prospero from the cell cortex of GMCs after cytokinesis. Both activities are essential during the localization and segregation of Prospero. Our study raises further questions: Miranda is itself asymmetrically localized—what proteins tether it to the basal cortex of neuroblasts? And what proteins regulate *miranda* so that it releases Prospero in the GMC once cytokinesis is complete? Answers to these questions should clarify how intrinsically programmed daughter cells can follow divergent developmental pathways^{18–20}. □

Methods

Cloning of the *miranda* gene. In two-hybrid screening¹⁰, the hybrid bait consisted of the yeast GAL4-binding domain and the *prospero* coding sequence spanning amino acids 3–1,237 (ref. 7). We screened 6×10^6 clones of a *Drosophila* embryonic cDNA library for clones that activated the lacZ reporter gene in combination with the bait. We subsequently tested the interaction of those clones with the control bait. The control bait lacks the 31 amino acids 871–901 in the *prospero* gene, which is the domain of Prospero that is necessary for asymmetric localization of Prospero *in vivo*⁷. We identified a single species of clone that did not activate the lacZ reporter in combination with the control bait. Using this clone as a probe, we isolated full-length *miranda* cDNA.

Immunohistochemistry. We raised rabbit antisera against the C-terminal polypeptide of the Miranda protein, SPPKQVVKARNI, and used monoclonal antibody MR1A for staining Prospero⁹, rabbit anti-Eve polyclonal serum to follow Eve protein expression, monoclonal antibody BP102 to follow axonal tracts, and TOTO3 (Molecular Probes) to visualize chromosomes⁷. We exam-

ined stained embryos using either a confocal microscope (MRC1024; BioRad) or a Zeiss Axioplan compound microscope.

Identification of *miranda* mutations. We used polytene *in situ* hybridization to map the *miranda* gene to 92C in the third chromosome and identified six *miranda* alleles from a stock of ethylmethanesulphonate-induced recessive lethal mutants (J.B.S. and C.O.D., unpublished data). This complementation group mapped to genetic map position 67.7 and failed to complement *DF (3R) ora*¹⁹ which uncovers the region of 92B1-3; 93C1-3 (ref. 21). Anti-Miranda antibodies fail to stain embryos homozygous for three alleles of this group: *miranda*^{L44}, *miranda*^{RR127} and *miranda*^{ZZ176}. To determine the open reading frame of two alleles, *miranda*^{RR127} and *miranda*^{ZZ176}, we isolated mRNA from heterozygous embryos and did polymerase chain reaction with reverse transcription (RT-PCR) to amplify *miranda* cDNA. We used five sets of PCR primers to cover the entire *miranda* open reading frame and sequenced more than 15 subclones of PCR fragments generated from each primer set.

Rescue experiment. We used the pUAST vector to construct the UAS-*miranda* transgene²², then introduced this construct into heterozygous *miranda*^{ZZ176} flies by transformation and crossed males of this genotype to heterozygous *miranda*^{ZZ176} females carrying the GAL4 gene driven by a ubiquitous promoter from the *armadillo* gene²³. We collected embryos from this cross and triple-stained them with anti-Miranda, anti-Prospero, and anti β -galactosidase antibodies (Promega). We identified *miranda*^{ZZ176} homozygous embryos based on the absence of *lacZ* expression from the balancer chromosome TM3, P[ry⁺, ftz-lacZ]. We identified embryos carrying both the UAS-*miranda* gene and the *arm-GAL4* gene by ubiquitous *miranda* expression.

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- Horvitz, H. R. & Herskowitz, I. Mechanisms of asymmetric cell divisions: two Bs or not two Bs, that is the question. *Cell* **68**, 237–255 (1992).
- Jan, Y. N. & Jan, L. Y. Maggot's hair and bug's eye: role of cell interactions and intrinsic factors in cell fate specification. *Neuron* **14**, 1–5 (1995).
- Campos-Ortega, J. A. Numb diverts Notch pathway off the Tramtrack. *Neuron* **17**, 1–4 (1996).
- Doe, C. Q., Chu-LaGriff, Q., Wright, D. M. & Scott, M. P. The *prospero* gene specifies cell fate in the *Drosophila* central nervous system. *Cell* **65**, 451–465 (1991).
- Vassini, H., Grell, E., Wolff, E., Bier, E., Jan, L. Y. & Jan, Y. N. *prospero* is expressed in neuronal precursors and encodes a nuclear protein that is involved in the control of axonal outgrowth in *Drosophila*. *Cell* **67**, 941–953 (1991).
- Matsuzaki, F., Koizumi, K., Hama, C., Yoshioka, T. & Nabeshima, Y. Cloning of the *Drosophila prospero* gene and its expression in ganglion mother cells. *Biochem. Biophys. Res. Commun.* **182**, 1326–1332 (1992).
- Hirata, J., Nakagoshi, H., Nabeshima, Y. & Matsuzaki, F. Asymmetric segregation of the homeodomain protein Prospero during *Drosophila* development. *Nature* **377**, 627–630 (1995).
- Fields, S. & Song, O. A novel genetic system to detect protein–protein interactions. *Nature* **340**, 245–247 (1989).
- Spana, E. & Doe, C. Q. The *prospero* transcription factor is asymmetrically localized to the cell cortex during neuroblast mitosis in *Drosophila*. *Development* **121**, 3187–3195 (1995).
- Knoblich, J. A., Jan, L. Y. & Jan, Y. N. Localization of Numb and Prospero reveals a novel mechanism for asymmetric protein segregation during mitosis. *Nature* **377**, 624–627 (1995).
- Uemera, T., Shepherd, S., Ackerman, L., Jan, L. Y. & Jan, Y. N. *numb*, a gene required in determination of cell fate during sensory organ formation in *Drosophila* embryos. *Cell* **5**, 349–360 (1989).
- Rhyu, M. S., Yan, L. Y. & Jan, Y. N. Asymmetric distribution of Numb protein during division of the sensory organ precursor cell confers distinct fates to daughter cells. *Cell* **76**, 477–491 (1994).
- Spana, E., Kopczynski, C., Goodman, C. S. & Doe, C. Q. Asymmetric localization of Numb autonomously determines sibling neuron identity in the *Drosophila* CNS. *Development* **121**, 3489–3494 (1995).
- Doe, C. Q., Hiromi, Y., Gehring, W. J. & Goodman, C. S. Expression and function of the segmentation gene *fushi tarazu* during *Drosophila* neurogenesis. *Science* **239**, 170–175 (1988).
- Doe, C. Q., Smouse, D. & Goodman, C. S. Control of neuronal fate by the *Drosophila* segmentation gene *even-skipped*. *Nature* **333**, 376–378 (1988).
- Doe, C. Q. Molecular markers for identified neuroblasts and ganglion mother cell in the *Drosophila* embryonic central nervous system. *Development* **116**, 855–863 (1992).
- Broadus, J., Skeath, J. B., Spana, E. P., Bossing, T., Technau, G. & Doe, C. Q. New Neuroblast marker and the origin of the aCC/pCC neurons in the *Drosophila* central nervous system. *Mech. Dev.* **53**, 393–402 (1995).
- Kraut, R., Chia, W., Jan, L. Y., Jan, Y. N. & Knoblich, J. A. Role of *inscuteable* in orienting asymmetric cell divisions in *Drosophila*. *Nature* **383**, 50–55 (1996).
- Eaton, S. & Simons, K. Apical, basal, and lateral cues for epithelial polarization. *Cell* **82**, 5–8 (1995).
- Lin, H. & Schagat, T. Neuroblasts: a model for the asymmetric division of stem cells. *Trends Genet.* **13**, 33–39 (1997).
- O'Tousa, J. E., Leonard, D. S. & Pak, W. L. Morphological defects in *ora*^{TRK} photoreceptors caused by mutation in R1-6 opsin gene of *Drosophila*. *J. Neurogenet.* **6**, 41–52 (1989).
- Brand, A. & Perrimon, N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401–415 (1993).
- Vincent, J.-P., Girdham, C. H. & O'Farrell, P. H. A cell-autonomous, ubiquitous marker for the analysis of *Drosophila* genetic mosaics. *Dev. Biol.* **164**, 328–331 (1994).

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Antiproliferative action of interferon- α requires components of T-cell-receptor signalling

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Signal transduction through both cytokine and lymphocyte antigen receptors shares some common pathways by which they initiate cellular responses, such as activation of mitogen-activated protein kinase(s)^{1,2}. However, other signalling components appear to be uniquely coupled to each receptor. For example, the interferon receptors transduce regulatory signals through the JAK/STAT pathway, resulting in an inhibition of growth and of antiviral effects, whereas this pathway apparently plays no role in T-cell-receptor (TCR)-dependent gene expression^{3,4}. Conversely, signal transduction through the TCR requires the tyrosine kinases Lck and ZAP-70 and the tyrosine phosphatase CD45 (ref. 5). Here we show that, unexpectedly, transmission of growth-inhibitory signals by interferon- α (IFN- α) in T cells requires the expression and association of CD45, Lck and ZAP-70 with the IFN- α -receptor signalling complex.

To determine whether any of the components required for TCR engagement also associate with the IFN- α receptor, cellular extracts were prepared from either untreated or IFN- α -treated fresh human peripheral blood lymphocytes (PBLs) obtained from normal donors or Jurkat T cells. The extracts were immunoprecipitated with an antibody that recognizes the α -subunit of the IFN- α receptor (IFN α R1), and the immunoprecipitates assayed by western blot analysis using antisera that recognize CD45, Lck or ZAP-70 (Fig. 1a). IFN- α caused a rapid, ligand-dependent interaction of CD45, ZAP-70 and Lck with IFN α R1. Immunoprecipitation with isotype-matched control antibody showed that the interaction was specific (Fig. 1a, lane 7). To ensure that equal amounts of IFN α R1 were in each sample, aliquots were analysed for the presence of the IFN α R1 receptor (Fig. 1a, lower panel). Immunoprecipitation with ZAP-70, CD45 or Lck antiserum, followed by blotting to test for the presence of IFN α R1, gave similar results (data not shown). Furthermore, ZAP-70 becomes tyrosine-phosphorylated as a result of incubation of cells with IFN- α (Fig. 1b). This finding parallels the observed tyrosine-phosphorylation of the enzyme that occurs after engagement of the TCR⁵.

To test whether the antiproliferative effects of IFN- α are regulated by the expression of ZAP-70, Lck or CD45, we used Jurkat cell variants that do not express these enzymes^{6–9}. Wild-type and Jurkat cell variants in the log phase of growth were incubated with or without IFN- α for 72 h and aliquots taken for counting. Parental cells cultured with IFN- α showed a 40–50% inhibition in cell

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growth, which is typical of the antiproliferative effects of IFN- α on a variety of cultured cells (Fig. 2a). However, cells lacking CD45 (CD45⁻), Lck (Lck⁻) or ZAP-70 (ZAP-70⁻) were markedly resistant to the growth inhibition exerted by this cytokine. These results were confirmed by examining ³H-thymidine incorporation (data not shown). All concentrations up to 2×10^4 units ml⁻¹ of IFN- α were ineffective in inhibiting growth in cells defective in CD45, Lck or ZAP-70. Like Jurkat cells, the human T-cell line HPB-ALL CD45-negative variant¹⁰ was also insensitive to the growth-inhibitory effects of IFN- α (Fig. 2a, lanes 17–20). In Jurkat variant lines in which CD45, Lck or ZAP-70 were re-expressed, the antiproliferative response to IFN- α was restored to that seen in wild-type cells (Fig. 2a: labelled CD45+, ZAP70+ and LCK+). Cells deficient in ZAP-70 and reconstituted with a kinase-inactive form of the enzyme (ZAP70 KD) were also resistant to the antiproliferative action of IFN- α , indicating that the kinase activity of ZAP-70 is required for growth arrest. Apoptosis was not seen in Jurkat cells treated with IFN- α , as assayed by DNA fragmentation, whereas OKT3, which stimulates signalling through the TCR, did induce DNA fragmentation (Fig. 2b). Similar results were seen in flow cytometry, which we

used to analyse the cell cycle (data not shown).

In contrast to the requirement for CD45, Lck and ZAP-70 for IFN α -mediated growth arrest, these enzymes had no effect on the ability of this cytokine to regulate the JAK/STAT signalling cascade or to protect Jurkat cells from infection by measles virus. IFN- α activation of the JAK/STAT pathway was assessed by the ability of tyrosine-phosphorylated Stat1 and Stat2 to bind to an interferon-stimulated response element (ISRE) and form an ISGF3 transcription complex⁴. Incubation with IFN- α of either wild-type (WT) Jurkat cells or cells deficient in CD45, Lck or ZAP-70 induced similar amounts of ISGF3 (Fig. 3a). IFN- α stimulated formation of Stat1-containing complexes which bind to interferon gamma activation sequence (GAS) elements, which was also identical in all cell lines (data not shown). Similarly, IFN- α induced Stat1- and/or Stat2-dependent RNAs, whose activation uses an ISRE (ISG15 and ISG54) or a GAS element (interferon regulatory factor 1, IRF-1, or guanylate binding protein, GBP) and which were unaffected by the lack of expression of CD45, Lck or ZAP-70 (Fig. 3b). Furthermore, IFN- α inhibited measles virus replication to the same extent in both the wild-type and Jurkat variants. Thus, the loss of the antiproliferative

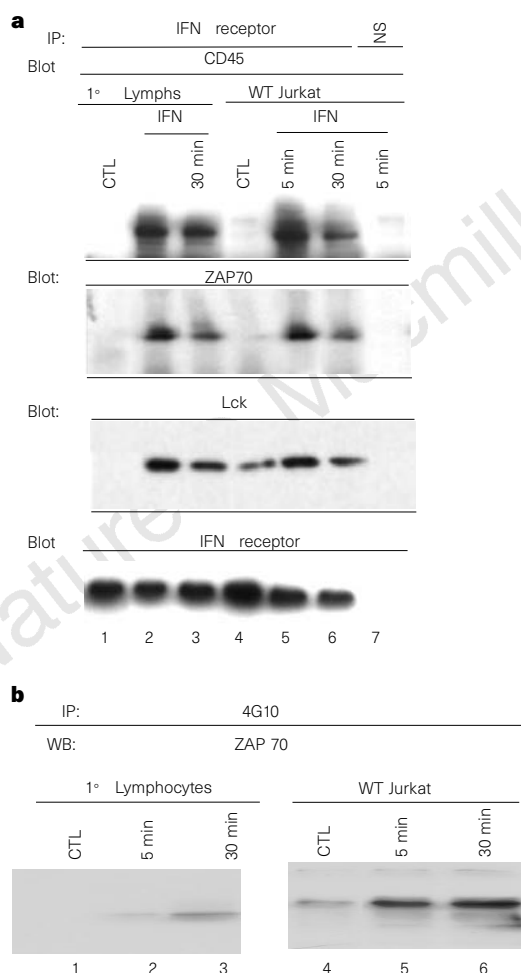


Figure 1 IFN- α stimulates association of CD45, Lck and ZAP-70 with the IFN- α receptor (IFN α R1) and ligand-induced tyrosine-phosphorylation of ZAP-70. **a**, Primary (1°) human peripheral blood lymphocytes (lymphs) or wild-type (WT) Jurkat cells were incubated without (CTL) or with IFN- α (10^3 units ml⁻¹) for 5 or 30 min. Immunoprecipitates (IP) with IFN α R1 antibody were resolved by SDS-PAGE and immunoblotted with anti-CD45 (top), ZAP-70 (second panel) or Lck (third panel). Blots were reprobed for IFN α R1 to ensure comparable loading (bottom panel). An isotype-matched monoclonal antibody was used in lane 7. **b**, Cellular extracts prepared from primary lymphocytes or Jurkat cells were immunoprecipitated with 4G10 anti-phosphotyrosine and blots were probed for ZAP-70.

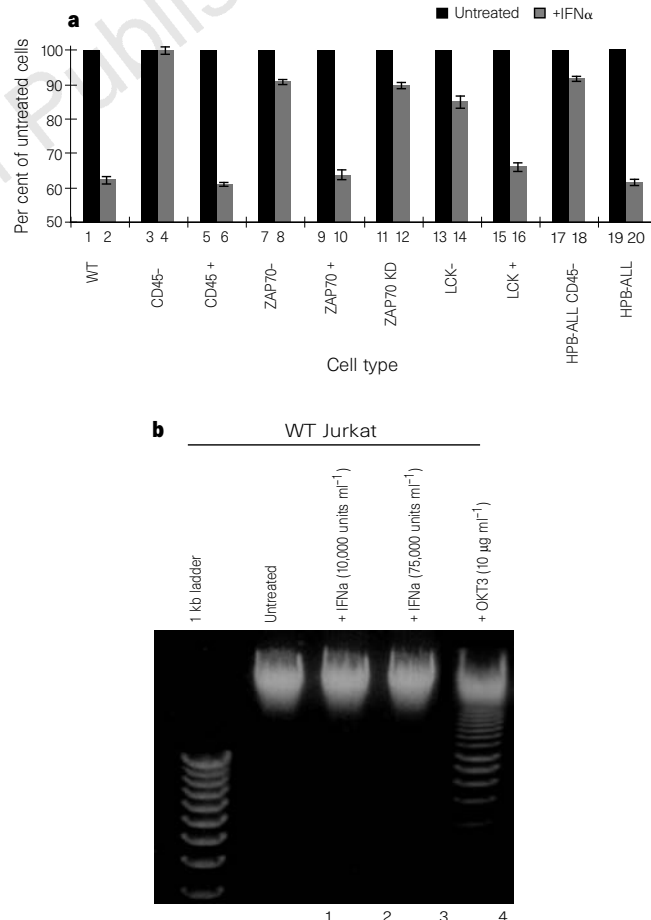


Figure 2 IFN- α -stimulated inhibition of cell growth is absent in Jurkat cells which do not express CD45, Lck or ZAP-70. **a**, Jurkat cells were incubated without or with 10^4 units ml⁻¹ IFN- α and cells were counted after 72 h. The number of viable cells in IFN- α -treated samples was divided by the number of cells in the untreated pair. Standard error bars were determined from 5 experiments. CD45+, ZAP70+ and Lck+ (lanes 5, 6, 9, 10, 15, 16) indicate those cells that have been reconstituted with the enzyme that was not expressed in the mutated cell lines. ZAP70KD corresponds to the ZAP-70-negative line in which the catalytically inactive form of the protein was expressed (lanes 11, 12). **b**, DNA fragmentation assay on either untreated cells (lane 1) or cells exposed to 10^4 units ml⁻¹ (lane 2) or 7.5×10^4 units ml⁻¹ (lane 3) IFN- α or with OKT3 (10μ g ml⁻¹) for 72 h (lane 4).

Table 1 Measles virus replication in parental and mutant Jurkat cell lines

Cells	Titre (TCID ₅₀ ml ⁻¹)		% Reduction in titre
	- IFN-α	+ IFN-α*	
Wild type	5.60	4.70	87
Lck -	6.20	4.95	94
CD45 -	5.45	3.95	97
ZAP 70-	6.92	4.23	>99

5 × 10⁵ cells were resuspended in RPMI ± 10⁴ U ml⁻¹ IFN-α 24 h prior and during infection with MV at a multiplicity of infection of 0.5–1 per cell. MV-infected and control cells were collected 72 h post-infection. Individual aliquots of IFN-α-treated cell pellets were incubated with rabbit anti-IFN-α antibody at 1:50 for 1 h at 4°C prior to titration on Vero cell monolayers. Titres are expressed as the reciprocal of the log₁₀ TCID₅₀ ml⁻¹ observed at day 6. Measles virus (MV): low passage Edmonston wild-type (EdHek/V₄PP₃Vero₄).

* Rabbit anti-IFN-α was added to MV-infected cells that were not treated with IFN-α at a final concentration of 1:500 to inhibit the effects of endogenous interferon production.

effects of IFN-α, but not of its activation of the JAK/STAT pathway or its antiviral action, is associated with lack of expression of CD45, Lck or ZAP-70 and the kinase activity of ZAP-70, is required for the antiproliferative actions of this cytokine.

TCR activation requires a sequential series of reactions initiated by CD45-induced dephosphorylation of Lck, followed by its activation and recruitment of ZAP-70 into the signalling complex, where it is activated. To determine whether IFN-α-induced association of ZAP-70, Lck or CD45 with IFNαR1 was also dependent upon a sequential requirement of these TCR components, we analysed the association of ZAP-70, Lck or CD45 with IFNαR1 in the variant cells (Fig. 4). IFN-α-induced association of ZAP-70 with IFNαR1 was not observed in CD45- or Lck- cells (Fig. 4a). In cells that contained no CD45, IFN-α failed to cause an association of Lck with the receptor, but in cells lacking ZAP-70, Lck did associate with IFNαR1 (Fig. 4b). In cells that do not express ZAP-70 or Lck, there was inducible association of CD45 with IFNαR1 (Fig. 4c). ZAP-70-negative cells

reconstituted with kinase-inactive ZAP-70 also showed an induced association of kinase-inactive ZAP-70 with IFNαR1 (Fig. 4d). It thus appears that, in a way analogous to activation of the TCR, a sequential series of interdependent events is needed for those T-cell signalling components to participate in the antiproliferative actions of IFN-α. A model indicating how the TCR components could be associated with IFNαR1 is shown in Fig. 5.

Expression of CD45, Lck and ZAP-70, and presumably their association with IFNαR1, is required for the antiproliferative actions of IFN-α in both Jurkat and HPB-ALL-transformed T-cell lines. Although activation of Lck and ZAP-70 through the TCR is associated with cell development, in the context of IFN-α stimulation of these kinases they may also contribute to the antiproliferative actions of this cytokine.

The role of the TCR components in the action of IFN-α in T cells suggests that in other cell types that are growth-arrested by IFN-α,

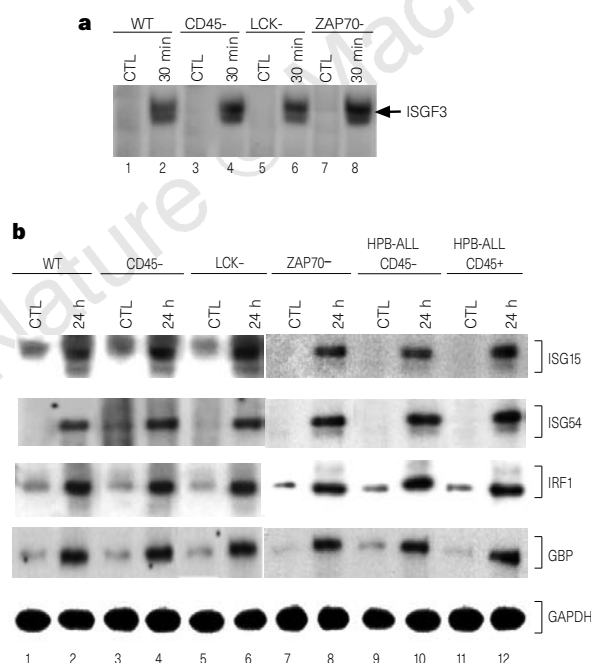


Figure 3 IFN-α-induced activation of the JAK/STAT pathway is identical in wild-type (WT) and variant Jurkat cells. **a**, Cells were incubated in the absence (lanes 1, 3, 5 and 7) or in the presence of IFN-α (10³ units ml⁻¹) (lanes 2, 4, 6 and 8) for 30 min and ISGF3 formation was assayed by EMSA with an ISRE probe. Cell lines deficient in the expression of CD45 are labelled (CD45-, lanes 3 and 4), Lck (Lck-, lanes 5 and 6) and ZAP-70 (ZAP70-, lanes 7 and 8). **b**, Total cellular RNA was isolated and RNase protection assays were done with cDNA probes corresponding to IFN-α-induced genes ISG15, ISG54, IRF-1 and GBP¹¹. A probe corresponding to GAPDH was included in the hybridization to ensure equal amounts of RNA were present in each sample.

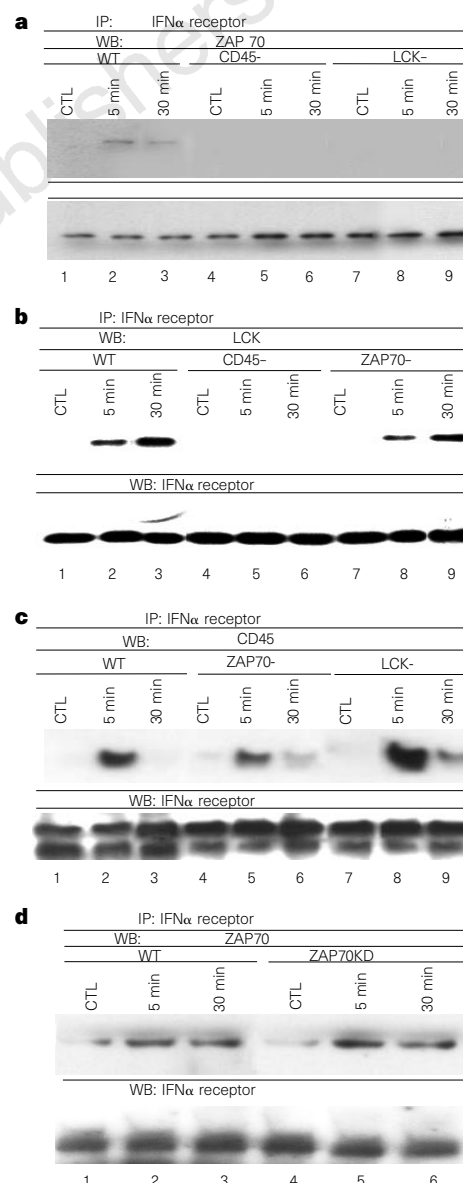


Figure 4 IFN-α-stimulated association of CD45, Lck and ZAP-70 with IFNαR1 in Jurkat cells. Parental Jurkat cells and Jurkat cell lines lacking CD45, Lck or ZAP-70 or lines reconstituted with kinase-inactive ZAP-70 were incubated in the absence or presence of IFN-α for 5 or 30 min. Cellular extracts were prepared and incubated with antibody to IFNαR1 as described for Fig. 1. The resulting blots were probed for the presence of ZAP-70 (**a** and **d**), Lck (**b**) CD45 (**c**). To ensure equal loading, each blot was probed for the presence of IFNαR1 (bottom panels).

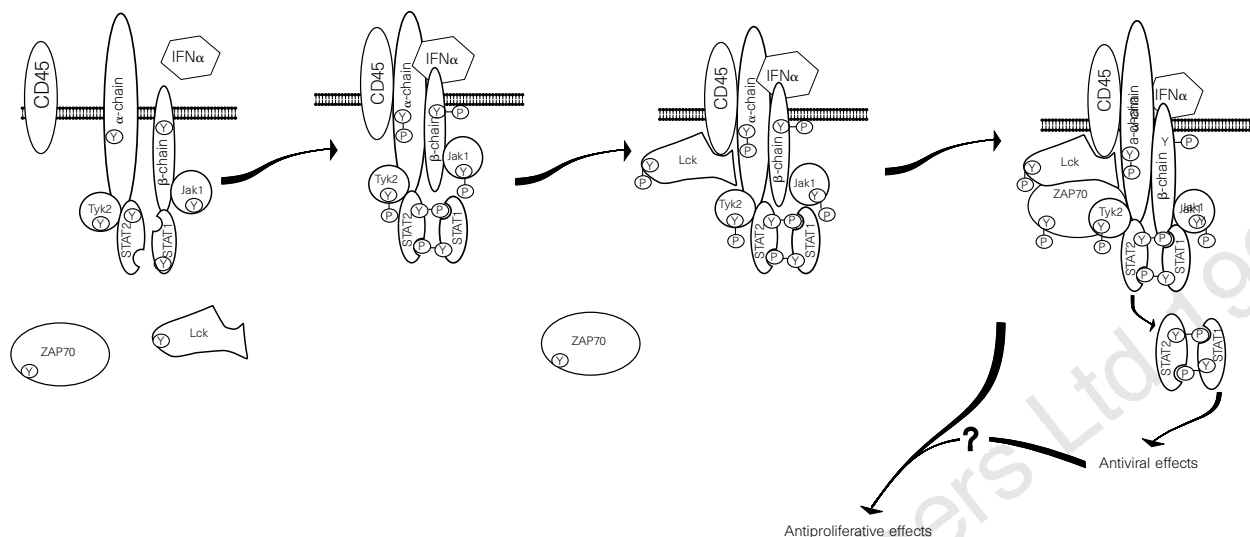


Figure 5 Representation of the distinct biological effects mediated by IFN- α in T cells. IFN- α binding to its receptor induces association of CD45 with the signalling complex, as well as tyrosine phosphorylation of Jak1, Tyk2, Stat1 and

Stat2. Lck and then ZAP-70 are subsequently recruited to the signalling complex and Lck and ZAP-70 are also tyrosine-phosphorylated.

additional pathway(s) must be considered as potentially important mediators of the antiproliferative effects of this cytokine, and activation of the JAK/STAT pathway alone cannot always determine the biological response to IFN- α . The fact that many other cytokines apart from the IFNs activate the JAK/STAT signalling cascade indicates that some other biological modifiers may use unexpected kinases and phosphatases to exert their distinct biological actions. □

Methods

Cells and reagents. Human lymphocytes were obtained by leukaphoresis of normal volunteers¹¹. Jurkat and HPB-ALL cell lines were cultured in RPMI-1640 containing 10% fetal calf serum. The characterization of the CD45-negative (J45.01) and the Lck-negative Jurkat subclones has been described^{6,7}. The ZAP-70 Jurkat subclone p116 was isolated during a screen for mutant cells with defects in pervanadate-induced Ca^{2+} mobilization⁹. Monoclonal antibodies were from Transduction Laboratories; 4G10 agarose beads were from Upstate Biotechnology; recombinant human IFN- α was from Hoffmann-LaRoche.

Electrophoretic mobility shift assay (EMSA). EMSAs were done using whole-cell extracts prepared with Triton-containing buffers as described¹¹.

Immunoprecipitations. Cell lysates (350 μg) were prepared with Triton X-100-containing buffers and were incubated with antibodies against the α -subunit of the IFN α / β receptor¹. Immune complexes were immunoblotted with anti-ZAP-70, anti-Lck or anti-CD45 and detected using ECL.

Antiviral and antiproliferative assays. Replication of measles virus (Edmonston strain) was determined using 1 and 0.1 multiplicity of infection (MOI) per cell. Cell-associated virus and supernatant fluids were titrated on VERO cell monolayers. The cytopathic effect was evaluated after 6 d and confirmed by staining with crystal violet.

Antiproliferative and DNA fragmentation assays. Cell lines in log growth phase were cultured in the absence or presence of IFN- α at 10^3 – 10^4 units per ml. Forty-eight or 72 h after addition of IFN- α , samples were counted for cell number or pulsed with [^3H]thymidine for 8 h before collection. DNA was analysed for fragmentation on 1% agarose gels.

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- David M. *et al.* Requirement for MAP kinase (ERK2) activity in interferon α / β -stimulated gene expression through Stat proteins. *Science* **269**, 1721–1723 (1995).
- Weiss, A. & Littman, D. R. Signal transduction by lymphocyte antigen receptors. *Cell* **76**, 263–274 (1994).
- Beadling, C. *et al.* Activation of JAK kinases and STAT proteins by interleukin-2 and interferon α , but not the T cell antigen receptor, in human T lymphocytes. *EMBO J.* **13**, 565–5615 (1994).
- Larner, A. C. & Finbloom, D. S. Protein tyrosine phosphorylation as a mechanism which regulates cytokine activation of early response genes. *Biochim. Biophys. Acta* **1266**, 278–287 (1995).

- Weiss, A. & Littman, D. R. Signal transduction by lymphocyte antigen receptors. *Cell* **76**, 263–274 (1994).
- Koretzky, G. A., Picus, J. T., Thomas, M. L. & Weiss, A. Tyrosine phosphatase CD45 is essential for coupling T-cell receptors to the phosphatidylinositol pathway. *Nature* **346**, 66–68 (1990).
- Straus, D. B. & Weiss, A. Genetic evidence for the involvement of the Lck tyrosine kinase in signal transduction through the T cell receptor. *Cell* **70**, 585–593 (1992).
- Weaver, C. T., Pingel, J. T., Nelson, J. O. & Thomas, M. T. CD8⁺ T-cell clones deficient in the expression of the CD45 protein tyrosine phosphatase have impaired responses to T-cell receptor stimuli. *Mol. Cell. Biol.* **11**, 4415–4422 (1991).
- Williams, B. L. *et al.* T cell antigen receptor signaling function in a ZAP-70-negative Jurkat cell line. *Mol. Cell. Biol.*, submitted.
- Shiroo, M., Goff, L., Biffen, M., Shivan, E. & Alexander, D. CD45 tyrosine phosphatase-activated p59^{lck} couples the T cell antigen receptor to pathways of diacylglycerol production, protein kinase C activation and calcium influx. *EMBO J.* **11**, 4887–4897 (1992).
- Larner, A. C. *et al.* Tyrosine phosphorylation of DNA binding proteins by multiple cytokines. *Science* **261**, 1730–1733 (1993).

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Cdc42 and Rac1 induce integrin-mediated cell motility and invasiveness through PI(3)K

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Transformation of mammary epithelial cells into invasive carcinoma results in alterations in their integrin-mediated responses to the extracellular matrix, including a loss of normal epithelial polarization and differentiation, and a switch to a more motile, invasive phenotype. Changes in the actin cytoskeleton associated with this switch suggest that the small GTPases Cdc42 and Rac, which regulate actin organization^{1,2}, might modulate motility and invasion. However, the role of Cdc42 and Rac1 in epithelial cells, especially with respect to integrin-mediated events, has not been well characterized. Here we show that activation of Cdc42 and Rac1 disrupts the normal polarization of mammary epithelial

cells in a collagenous matrix, and promotes motility and invasion. This motility does not require the activation of PAK, JNK, p70 S6 kinase, or Rho, but instead requires phosphatidylinositol-3-OH kinase (PI(3)K). Further, direct PI(3)K activation is sufficient to disrupt epithelial polarization and induce cell motility and invasion. PI(3)K inhibition also disrupts actin structures, suggesting that activation of PI(3)K by Cdc42 and Rac1 alters actin organization, leading to increased motility and invasiveness.

Consistent with their well-differentiated phenotype, T47D mammary epithelial cells organize into multicellular, polarized tubule structures when cultured in three-dimensional collagen gels (Fig. 1a). Formation of these structures requires the $\alpha 2\beta 1$ integrin³. Stable expression of constitutively active Cdc42(12V) prevented the formation of these polarized structures, and the cells grew instead as disorganized sheets of individual cells (Fig. 1b). Overexpression of wild-type Rac1 or constitutively active Rac1(61L) also prevented polarization of the tubule structures (Fig. 1c, d). Cells overexpressing wild-type Cdc42 were the same as control cells (data not shown). T47D cells were also transfected with guanine-nucleotide exchange factors, which promote the GTP-bound state of endogenous Rho family members. Expression of activated Dbp, an exchange factor for both Cdc42 and RhoA⁴, promoted a phenotype consistent with that noted for Cdc42(12V); the cells grew in disorganized sheets (Fig. 1e), and formed foci in collagen gel cultures. The effect of Dbp was probably a result of effects on Cdc42 and not on RhoA, as expression of either activated RhoA(63L) or the RhoA-specific exchange factors Lfc and Lsc⁵ enhanced the formation of polarized tubule structures (data not shown). These observations suggest that the regulation of Cdc42 and Rac1 helps to establish epithelial polarity in response to the extracellular matrix. A role for Cdc42 in determining cellular polarity has been suggested for yeast⁶, *Drosophila*⁷ and T lymphocytes⁸, but has not to our knowledge been demonstrated in the formation of organized, polarized structures in mammalian epithelial cells.

Activated Cdc42(12V), Rac1 and Rac1(61L) also promoted a motile phenotype, as detected by a three- to fivefold increase in cell motility across collagen (Fig. 2a), whereas overexpression of wild-type Cdc42 had no effect on cell motility (Fig. 2a). The increased cell motility was mediated by integrins, as about half of the Cdc42(12V)-induced motility and all of the Rac1-induced motility was inhibited by an antibody against the $\alpha 2$ integrin subunit (Fig. 2b). The remainder of the Cdc42(12V)-induced motility was inhibited by an anti- $\beta 1$ integrin antibody (Fig. 2b), but not by an antibody to the $\alpha 3$ integrin subunit, another collagen receptor on these cells (data not shown). This motility is not caused by growth factors, as it occurred in the absence of serum.

The motility induced by Cdc42 and Rac1 differed in substratum specificity. Cdc42(12V)-expressing cells were more motile on collagens, but not on fibronectin (Fig. 2c). In contrast, Rac1 cells (Fig. 2c) and Rac1(61L) cells (data not shown) exhibited increased motility on all substrata tested, implying that Rac1 and Cdc42 might affect motility through different integrin subunits. These results also suggest that Cdc42-induced motility does not occur through effects on Rac1. Cdc42(12V) and Rac1 did not alter the expression of integrin subunits $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$ or $\alpha 6$, as determined by flow cytometry (data not shown). The adhesion of cells expressing Cdc42(12V) and Rac1 to collagen I, collagen IV, laminin or fibronectin was not altered (data not shown), suggesting that there were no obvious changes in integrin avidity.

Cdc42(12V), Rac1 and Rac1(61L) also induced significant increases in the invasiveness of mammary epithelial cells through collagen gels in response to a serum gradient (Fig. 2d). A role in T-lymphocyte and fibroblast invasion has been demonstrated for activated Rac1 and Tiam-1 (refs 9–11), a Rac1 exchange factor. However, such a role for Rac1 in epithelial cells or for Cdc42 in any cell type has not been shown previously. Thus activation of Cdc42 or Rac1 switches the response of mammary epithelial cells to collagenous matrices from a polarized, differentiated phenotype to a motile, invasive phenotype.

We investigated signalling pathways downstream of Cdc42 and Rac1 that might be responsible for the altered phenotype. Cdc42-GTP and Rac-GTP activate, through MLK3 (ref. 12) or another unidentified effector, a serine/threonine kinase cascade of MEKK, SEK/MEK4 and JNK/SAPK^{13,14}. Cdc42 and Rac-GTP also bind to and activate members of the PAK (p21-activated kinases) family. However, the activated Rac1(12V) effector domain mutants Rac1(12V)T35S and Rac1(12V)Y40H, which no longer bind to PAK and show diminished or no activation of JNK/SAPK^{15,16}, did not inhibit cell motility; indeed they increased it (Fig. 3a), suggesting that activation of PAKs or JNK is not involved in Rac-induced cell motility. Similar results were obtained for invasion (data not shown). In addition, cells expressing Cdc42(12V) and Rac1 were co-transfected with dominant-negative SEK/MEK4 (ref. 17), which diminished the activation of JNK by tumour necrosis factor (TNF)- α in T47D cells (data not shown), but had no effect on the motility of control, Cdc42(12V) or Rac1 cells (data not shown), confirming that JNK activation is not required for cell motility. These findings are consistent with similar studies that dissociate Cdc42 and Rac-induced actin polymerization from PAK and JNK activation^{15,16,18}. Rac1(12V)F37L, an effector-domain mutant that retains PAK binding activity but fails to bind other effectors such as POR1 and exhibits reduced transcription from cyclin D1, Jun or SRF promoters^{15,16}, also induced T47D cell motility (Fig. 3a) and invasion

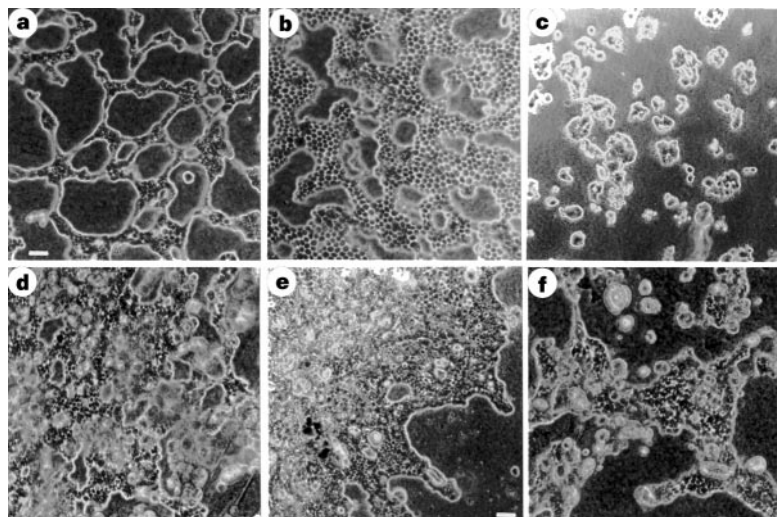


Figure 1 Expression of activated Cdc42, Rac1 or PI(3)K disrupts the organized polarization of mammary epithelial cells. Cells stably expressing vector only (a), Cdc42(12V) (b), Rac1 (c), Rac1(61L) (d), Dbp, a Cdc42/Rho exchange factor (e), or p110(K227E), a constitutively active PI(3)K (f) were cultured in three-dimensional collagen gels^{3,28}. Magnification: a–d, f, $\times 200$; e, $\times 100$. Scale bars: a, 60 μ m; e, 120 μ m.

(data not shown). These cells also exhibited increased spreading and lamellipodial formation on collagen (data not shown), a result that is consistent with their motile phenotype but differs from observations of F37 effector mutants in other cell types^{15,16,18}. All three Rac1(12V) effector-domain mutants showed reduced activation of Gal-Jun transcriptional activity compared with Rac(12V) in T47D cells as assayed by an *in vivo* reporter gene assay (data not shown), consistent with results obtained in other cell types¹⁶. These effector-domain mutants therefore allow us to exclude several potential

mechanisms by which Rac, and probably Cdc42, induces cell motility in mammary epithelial cells.

Cdc42- and Rac1- induced motility also seems to be independent of p70 S6 kinase activation¹⁹, because motility was not affected by rapamycin (30 nM), an indirect but specific inhibitor of p70 S6 kinase²⁰ (Fig. 3b). Similarly, Cdc42 or Rac1 activation of leukotrienes and subsequent activation of Rho²¹ did not cause the observed increase in cell motility, as nordihydroguaric acid (NDGA, 10 μ M), a 5-lipoxygenase inhibitor that inhibits Rho activation by Rac²¹, also had no effect on cell motility (Fig. 3b).

Cdc42-GTP and Rac-GTP activation of PI(3)K was considered because they bind to the p85 subunit of PI(3)K and are proposed to activate PI(3)K directly^{22,23}. Two specific inhibitors of PI(3)K, wortmannin (30 nM) and LY294002 (25 μ M), inhibited the motility of cells expressing Cdc42(12V), Rac1 and Rac1(61L) to control levels (Fig. 4a). Further, LY294002 diminished invasion of these cells through collagen (Fig. 4b). These results indicate that activation of PI(3)K is required downstream of Cdc42 and Rac1 for epithelial cell motility and invasion.

To further investigate the role of PI(3)K, T47D cells were transfected with the PI(3)K p110 catalytic subunit containing an activating point mutation, p110(K227E)²⁴. Expression of p110(K227E) induced a threefold increase in basal PI(3)K activity *in vivo* (Fig. 4c), and induced a three- to fourfold increase in both cell motility and invasion across collagen matrices (Fig. 4d). Expression of p110(K227E) also disrupted the organization of T47D cells into polarized structures in three-dimensional collagen gel culture (Fig. 1f). Therefore PI(3)K activation is not only necessary but also sufficient to convert epithelial cells from a polarized morphology to a motile, invasive phenotype.

Because PI(3)K is implicated in Ras-induced actin rearrangements²⁵, we investigated the effect of PI(3)K inhibition on actin polymerization during spreading of mammary epithelial cells. After 30 min of attachment to collagen, control cells had just begun to spread (Fig. 5a), whereas cells expressing Cdc42(12V) had produced elongated lamellipodia (Fig. 5b) and cells expressing Rac1(61L) had spread circumferentially with numerous stress

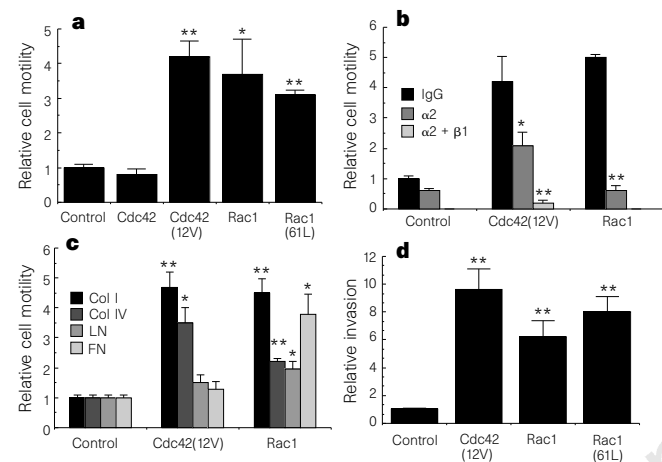


Figure 2 Cdc42 and Rac induce motility and invasiveness. **a**, Migration of cells across filters coated with collagen I. ** $P < 0.005$; * $P < 0.05$ relative to control migration. **b**, Cell motility is integrin mediated. Cells were preincubated with: control IgG, P1E6 (an anti- $\alpha 2$ integrin antibody; $\alpha 2$) or P1E6 + P4C10 (an anti- $\beta 1$ antibody; $\alpha 2 + \beta 1$). ** $P < 0.005$; * $P < 0.05$ versus IgG. **c**, Migration of cells across collagen I (Col I), collagen IV (Col IV), laminin (LN) or fibronectin (FN). ** $P < 0.005$; * $P < 0.05$, versus control migration for each substratum. **d**, Invasion of cells through a collagen gel in response to a serum gradient. ** $P < 0.005$ relative to control invasion.

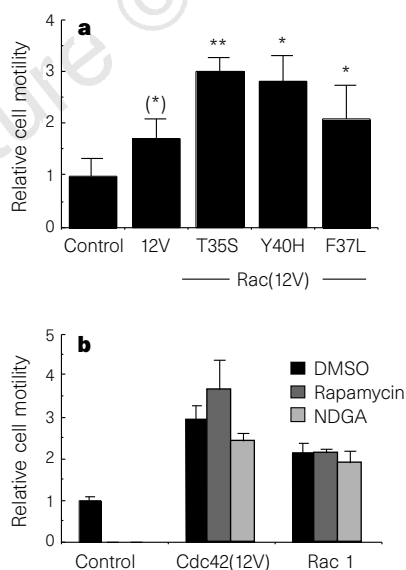


Figure 3 Signaling pathways and effectors downstream of Cdc42 and Rac. **a**, Migration across collagen of cells stably transfected with vector alone, activated Rac1(12V), or Rac1(12V) constructs with additional mutations in the effector-binding loop: T35S, Y40H or F37L. ** $P < 0.005$; * $P < 0.05$, (*) $P = 0.08$ compared to control migration. **b**, Motility of cells treated with rapamycin (an inhibitor of p70 S6 kinase), or NDGA (an inhibitor of 5-lipoxygenase) was not statistically different from DMSO treatment for each cell line ($P > 0.20$). Mean $\pm$ s.d. for two experiments.

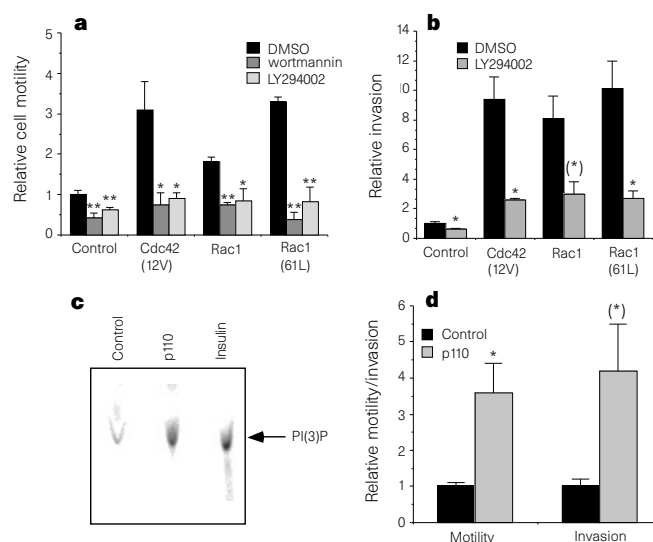


Figure 4 PI(3)K mediates Cdc42- and Rac-induced cell motility and invasion. **a**, PI(3)K inhibitors wortmannin (30 nM) or LY294002 (25 μ M) inhibit cell migration across collagen. ** $P < 0.005$; * $P < 0.05$ compared with DMSO-treated cells. **b**, PI(3)K inhibition by LY294002 inhibits invasion through a collagen gel. ** $P < 0.005$; * $P < 0.05$, (*) $P = 0.07$. **c**, *In vitro* kinase activity of PI(3)K immunoprecipitated from unstimulated cells expressing control vector or activated PI(3)K, p110(K227E), or control cells stimulated with insulin. PI(3)K activity with p110 or insulin is increased 2.9- and 3.4-fold, respectively, compared with the control. **d**, migration and invasion of control cells or cells expressing p110(K227E). ** $P < 0.05$, (*) $P = 0.07$.

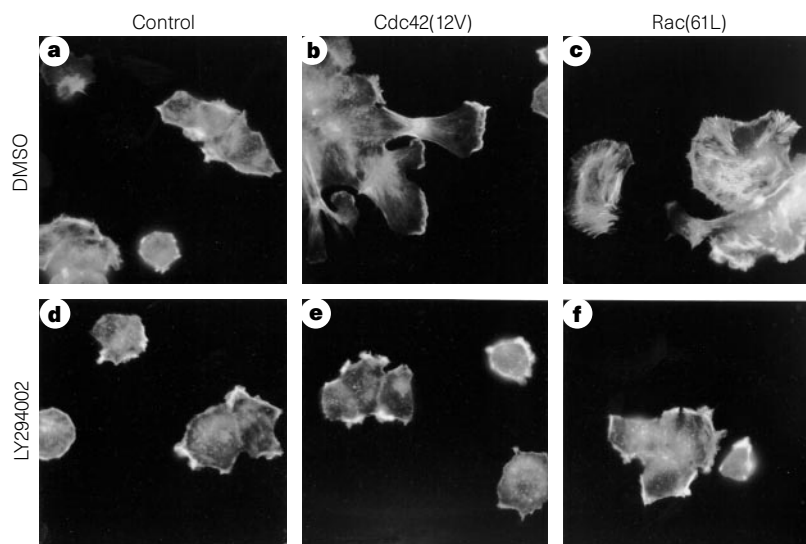


Figure 5 PI(3)K inhibition disrupts Cdc42- and Rac-induced spreading and formation of actin structures. Control (**a**, **d**) and cells expressing Cdc42(12V) (**b**, **e**) and Rac(61L) (**c**, **f**) were detached, pretreated with DMSO (**a–c**) or LY294002 (25 μ M, **d–f**) for 20 min, and allowed to attach and spread on collagen for 30 min before fixation. Actin structures were visualized by staining with fluorescein-phalloidin.

fibres (Fig. 5c). Wortmannin (30 nM, data not shown) or LY294002 (25 μ M) dramatically inhibited the spreading of cells expressing Cdc42(12V) and Rac1(61L) (Fig. 5d–f). In addition, wortmannin or LY294002 inhibited the formation of lamellipodia and stress fibres in these cells, and the actin appeared condensed near the cell periphery (Fig. 5d–f). Thus enhanced spreading and lamellipodial formation on collagen require PI(3)K, correlating with cell motility and invasion.

Our results demonstrate that chronic activation of Cdc42 and Rac1 affects the integrin-mediated responses of epithelial cells to the extracellular matrix by disrupting the normal, polarized organization of these cells in collagenous matrices and promoting a motile, invasive phenotype. The effect of Cdc42 and Rac1 activation on cell phenotype mirrors their effects on increasing the formation of motile actin-based structures including lamellipodia. Our results further indicate that PI(3)K activation is sufficient to induce the motile phenotype, and is necessary for Cdc42- and Rac-induced cell motility and invasiveness, probably through its effects on the actin cytoskeleton. □

Methods

Cell lines. T47D cells (American Type Culture Collection) were transfected with constructs for wild-type Cdc42 and Rac1, or activated versions (Cdc42(12V) or Rac1(61L) that were expressed in pZIP, or with activated Rac1(12V) or Rac1(12V) constructs that had second mutations in the effector-binding loop converting Thr 35 to Ser, Tyr 40 to His, or Phe 37 to Leu, which were expressed in pCGT (gift from L. Van Aelst^{15,16}). Exchange factors, Dbs⁴, Lfc²⁶ and Lsc²⁷ had small activating N-terminal truncations and were haemagglutinin-tagged and expressed in pCTV3. For activated PI(3)K, cells were co-transfected with p110(K227E)²⁴ and pcDNA3 (20:1). Cells were selected in G418 or hygromycin-containing growth medium as described³ and expanded as pools of stably transfected cells. Control cells were transfected with the appropriate vector alone and selected in parallel with the other cells. For double transfectants, stable cell lines were secondarily transfected with pCGN or dominant-negative pEBG-SEK¹⁷ and pCGN (20:1), and selected in hygromycin-containing growth medium³. Expression of constructs was determined by immunoblotting. Expression of Rac1 was 1.5-fold greater than Rac1(61L) and 2.5-fold greater than Rac1(12V), which might explain differences in how potentially each induces motility. Rac(12V) effector-domain constructs were expressed at levels equal to that of Rac1(12V).

Cell motility and invasion. Motility and invasion across transwells coated from the underside with 50 μ g ml⁻¹ collagen, laminin or fibronectin were assessed as described^{3,28}. Motility in the absence of serum or growth factors was assayed after 16 h. For inhibitor assays, cells were pretreated for 20 min with DMSO (control), rapamycin (30 ng ml⁻¹), nordihydroguarectic acid (NDGA, 10 μ M), wortmannin (30 nM), or LY294002 (25 μ M), allowed to migrate

across collagen in the continued presence of the inhibitor, and assayed after 5 h. The effectiveness of rapamycin was confirmed by its ability to inhibit both basal and anisomycin-induced activation of p70 S6 kinase in T47D cells. Invasion across 2-mm collagen gels was assessed after 20 h on serum-starved cells in response to serum placed in the lower chamber of the transwell. Unless otherwise noted, values presented are mean $\pm$ s.e. of at least three experiments of duplicate wells normalized to control values. Data were analysed by performing one-tailed, paired and unpaired Student's *t*-tests with Instat software. Morphogenesis in three-dimensional collagen gels was assessed after 8 days as described^{13,28}.

PI(3)K activity. To determine PI(3)K activity, cells expressing control vector or p110(K227E) were serum-starved overnight, lysed as described²⁹, and PI(3)K immunoprecipitated with antibodies to either the p85 subunit or the p110 subunit (Santa Cruz); the results were the same with either antibody. As a positive control, cells were treated with 100 nM insulin for 10 min before lysis. Phosphorylation of phosphatidylinositol to phosphatidylinositol-3-phosphate was determined by an *in vitro* immune-complex kinase assay²⁹ and the product resolved by thin-layer chromatography on an oxylated silica gel 60 plate using CHCl₃:methanol:water:ammonium hydroxide (30:24:5:6:1) as the solvent system. The image was digitized with NIH Image software.

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- Ridley, A. J., Paterson, H. F., Johnston, C. L., Diekmann, D. & Hall, A. The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell* **70**, 401–410 (1992).
- Nobes, C. D. & Hall, A. Rho, Rac, and Cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* **81**, 53–62 (1995).
- Keely, P., Fong, A., Zutter, M. & Santoro, S. Alteration of collagen-dependent adhesion, motility, and morphogenesis by the expression of antisense α 2 integrin mRNA in mammary cells. *J. Cell Sci.* **108**, 595–607 (1995).
- Whitehead, I. P., Campbell, S., Rossman, K. L. & Der, C. J. Dbl family proteins. *Biochim. Biophys. Acta* **1332**, F1–F23 (1997).
- Glaven, J. A., Whitehead, I. P., Nomanbhoy, T., Kay, R. & Cerione, R. A. Lfc and Lsc oncoproteins represent two new guanine nucleotide exchange factors for the Rho GTP-binding protein. *J. Biol. Chem.* **271**, 27374–27381 (1996).
- Adams, A. E. M., Johnson, D. I., Longnecker, R. M., Sloat, B. F. & Pringle, J. R. Cdc42 and Cdc43, two additional genes involved in budding and the establishment of cell polarity in the yeast *Saccharomyces cerevisiae*. *J. Cell Biol.* **111**, 131–142 (1990).
- Eaton, S., Auvinen, P., Luo, L., Jan, Y. N. & Simons, K. Cdc42 and Rac1 control different actin-dependent processes in the *Drosophila* wing disc epithelium. *J. Cell Biol.* **131**, 151–164 (1995).
- Stowers, L., Yelon, D., Berg, L. J. & Chant, J. Regulation of the polarization of T cells toward antigen-presenting cells by Ras-related GTPase CDC42. *Proc. Natl Acad. Sci. USA* **92**, 5027–5031 (1995).
- Michiels, F., Habets, G. G. M., Stam, J. C., van der Kammen, R. & Collard, J. G. A role for Rac in Tiam1-induced membrane ruffling and invasion. *Nature* **375**, 338–340 (1995).
- van Leeuwen, F. N., van der Kammen, R. A., Habets, G. G. & Collard, J. G. Oncogenic activity of Tiam1 and Rac1 in NIH3T3 cells. *Oncogene* **11**, 2215–2221 (1995).
- Collard, J. G. Signaling pathways regulated by Rho-like proteins: A possible role in tumor formation and metastasis. *Int. J. Oncol.* **8**, 131 (1996).
- Teramoto, H. et al. Signaling from the small GTP-binding proteins Rac1 and Cdc42 to the c-Jun N-terminal Kinase/Stress-activated protein kinase pathway. *J. Biol. Chem.* **271**, 27225–27228 (1996).
- Minden, A., Lin, A., Claret, F. X., Abo, A. & Karin, M. Selective activation of the JNK signaling cascade and c-jun transcriptional activity by the small GTPases Rac and Cdc42Hs. *Cell* **81**, 1147–1157 (1995).
- Coso, O. A. et al. The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. *Cell* **81**, 1137–1146 (1995).
- Joneson, T., McDonough, M., Bar-Sagi, D. & Van Aelst, L. Rac regulation of actin polymerization and proliferation by a pathway distinct from Jun kinase. *Science* **274**, 1374–1376 (1996).
- Westwick, J. K. et al. Rac regulation of transformation, gene expression, and actin organization by

- multiple, PAK-independent pathways. *Mol. Cell Biol.* **17**, 1324–1335 (1997).
17. Yan, M. *et al.* Activation of stress-activated protein kinase by MEKK1 phosphorylation of its activator SEK1. *Nature* **372**, 798–800 (1994).
 18. Lamarche, N. *et al.* Rac and Cdc42 induce actin polymerization and G1 cell cycle progression independently of p65PAK and the JNK/SAPK MAP kinase cascade. *Cell* **87**, 519–529 (1996).
 19. Chou, M. M. & Blenis, J. The 70 kDa S6 kinase complexes with and is activated by the Rho family G proteins Cdc42 and Rac1. *Cell* **85**, 573–583 (1996).
 20. Blenis, J., Chung, J., Erikson, E., Alcorta, D. A. & Erikson, R. L. Distinct mechanisms for the activation of the RSK kinases/MAP2 kinase/pp90rsk and pp70 S6 kinase signaling systems are indicated by inhibition of protein synthesis. *Cell Growth Differ.* **2**, 279–285 (1991).
 21. Peppelenbosch, M. P. *et al.* Rac mediates growth factor-induced arachidonic acid release. *Cell* **81**, 849–856 (1995).
 22. Zheng, Y., Bagrodia, S. & Cerione, R. A. Activation of phosphoinositide 3-kinase activity by Cdc42Hs binding to p85. *J. Biol. Chem.* **269**, 18727–18730 (1994).
 23. Tolias, K. F., Cantley, L. C. & Carpenter, C. L. Rho family GTPases bind to phosphoinositide kinases. *J. Biol. Chem.* **270**, 17656–17659 (1995).
 24. Rodriguez-Viciana, P., Warne, P. H., Vanhasebroeck, B., Waterfield, M. D. & Downward, J. Activation of phosphoinositide 3-kinase by interaction with Ras and by point mutation. *EMBO J.* **15**, 2442–2451 (1996).
 25. Rodriguez-Viciana, P. *et al.* Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by Ras. *Cell* **89**, 457–467 (1997).
 26. Whitehead, I., Kirk, H., Tognon, C., Trigo-Gonzalez, G. & Kay, R. Expression cloning of Ifc, a novel oncogene with structural similarities to guanine nucleotide exchange factors and to the regulatory region of protein kinase C. *J. Biol. Chem.* **270**, 18388–18395 (1995).
 27. Whitehead, I. P. *et al.* Expression cloning of Ifc, a novel oncogene with structural similarities to the Dbl family of guanine nucleotide exchange factors. *J. Biol. Chem.* **271**, 18643–18650 (1996).
 28. Santoro, S. A. *et al.* Analysis of collagen receptors. *Methods Enzymol.* **245**, 147–183 (1994).
 29. Auger, K. R., Serunian, L. A., Soltoff, S. P., Libby, P. & Cantley, L. C. PDGF-dependent tyrosine phosphorylation stimulates production of novel polyphosphoinositides in intact cells. *Cell* **57**, 167–175 (1989).

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An extended microtubule-binding structure within the dynein motor domain

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Flagellar dynein was discovered over 30 years ago as the first motor protein capable of generating force along microtubules¹. A cytoplasmic form of dynein has also been identified which is involved in mitosis and a wide range of other intracellular movements² (reviewed in ref. 3). Rapid progress has been made on understanding the mechanism of force production by kinesins and myosins^{4–8}. In contrast, progress in understanding the dyneins has been limited by their great size (relative molecular mass 1,000K–2,000K) and subunit complexity. We now report evidence that the entire carboxy-terminal two-thirds of the 532K force-producing heavy chain subunit is required for ATP-binding activity. We further identify a microtubule-binding domain, which, surprisingly, lies well downstream of the entire ATPase region and is predicted to form a hairpin-like stalk. Direct ultrastructural analysis of a recombinant fragment confirms this model, and suggests that the mechanism for dynein force production differs substantially from that of other motor proteins.

Two or three extremely large (320K–400K) force-producing dynein 'head' domains are found per dynein molecule, each formed from a heavy-chain polypeptide which contains four ATP-binding P-loop motifs spaced ~300 amino acids apart within its central region (reviewed in refs 9 and 10). To investigate the dynein heavy-chain functional organization, we have expressed the entire wild-type polypeptide^{11,12} and an extensive series of point and truncation mutant fragments in COS7 cells. To assess the nucleotide-binding capability of the mutant and wild-type heavy chains,

we exposed cell lysates to ultraviolet light in the presence of vanadate, which cleaves the dynein heavy chain in the ATP-binding region¹³. The full-length, Flag-tagged rat cytoplasmic dynein heavy chain was cleaved normally (Figs 1, 2) and supported microtubule translocation in *in vitro* sliding assays¹². It also showed diffuse and punctate cell staining (Fig. 3a), similar to the pattern of endogenous dynein¹⁰.

The first P-loop element has been suggested to be the primary site of photocleavage and ATPase activity on the basis of the estimated size of the VO₄ photocleavage fragments^{14,15}. When we introduced a K-to-E point mutation within the first P-loop consensus site at residue 1,910, the mutant protein failed to photocleave (Fig. 1). In addition, the K1910E mutant heavy chain showed clear colocalization with microtubules (Fig. 3b, c), consistent with formation of a rigor-like complex.

A fragment of the *Dictyostelium* cytoplasmic dynein heavy chain corresponding to the C-terminal two-thirds has been observed by electron microscopy to contain the globular head domain and to

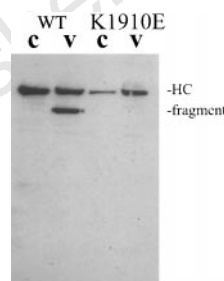


Figure 1 Effect of a dynein heavy-chain point mutation on VO₄-mediated photocleavage. Immunoblot showing VO₄-mediated photocleavage of recombinant Flag-tagged, full-length (WT) and K1910E point-mutant dynein heavy chain (HC) expressed in COS7 cells. Untreated control (c) and experimental samples (v) exposed to VO₄ and ultraviolet light. Electrophoresis was done on 6% polyacrylamide, and the dynein heavy chain was visualized using monoclonal anti-Flag antibody. Because the Flag epitope tag was located at the C terminus of the heavy chain, only the C-terminal photocleavage fragment was detected.

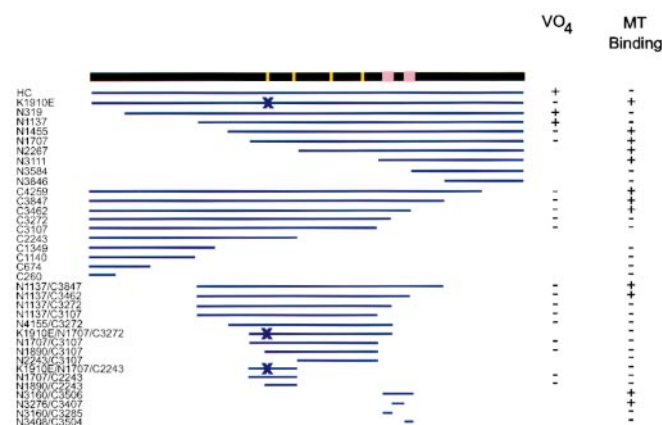


Figure 2 Summary of recombinant dynein heavy-chain behaviour in VO₄ photocleavage and microtubule binding assays. Diagram of the structural organization of the full-length wild-type dynein heavy chain is shown at the top. P-loop elements are indicated as yellow boxes, and predicted coiled-coil sequences flanking the microtubule-binding region are indicated by pink boxes. 'X' indicates the position of the K1910E point mutation. Results of VO₄-mediated photocleavage and microtubule co-localization are indicated on the right. Constructs that co-localized with microtubules did so in virtually all cells, with the exception of the K1910E construct, for which co-distribution was seen in only ~20% of cells. Mutant heavy chain expressed from constructs N1455 and N1707 appeared to be truncated at the C-terminus and could be detected using anti-dynein heavy chain but not anti-Flag antibody.

retain a functional photocleavage site¹⁶. To define more precisely the regions of the heavy chain required for photocleavage, we examined our series of deletion mutants. Surprisingly, VO₄-mediated photocleavage was lost when the protein was deleted from the N terminus to residue 1,455 (450 amino acids upstream of the first P-loop), or from the C terminus to residue 4,259 (Fig. 2). These results suggested that the globular head domain corresponds to the entire C-terminal two-thirds of the heavy chain and that any disruption of the head affects ATP binding and/or ATPase activity.

Results of immunofluorescence microscopy were consistent with this hypothesis. Fragments that retained sensitivity to VO₄-mediated photocleavage exhibited the diffuse distribution observed for the full-length heavy chain (Figs 2, 3d). However, when sensitivity to photocleavage was abolished by truncation of the heavy chain from the N or C terminus, striking microtubule colocalization and often microtubule bundling and reorganization were observed (Figs 2 and 3e, g, h), resembling overexpression of microtubule-associated proteins¹⁷. These observations suggested that inactivation of the ATPase site resulted in a rigor-like interaction between the dynein heavy chain and microtubules.

Analysis of the behaviour of 35 dynein heavy-chain fragments allowed us to map the microtubule-binding site (Figs 2, 3). Examination of N-terminal truncation mutants revealed that microtubule binding persisted with the removal of the first P-loop, and even the entire four-P-loop region (Figs 2, 3g). Binding was ultimately lost by deletion of amino acids 3,111–3,584, a region ~180 amino acids downstream from the fourth P-loop. Analysis of C-terminal deletions and of combined N- and C-terminal deletions implicated the same region in microtubule binding, within which fragments of 346 residues (N3160/C3506) and 131 residues (N3276/C3407) also clearly colocalized with microtubules (Figs 2 and 4i–l). Deletions of the K1910E point-mutant construct failed to identify additional microtubule-binding sites within the P-loop region (Fig. 2).

The microtubule-binding domain maps to a region found in all dynein heavy chains sequenced so far¹⁸, consisting in rat¹¹ of two predicted coiled-coil α -helices of 105 and 95 amino acids, respectively, separated by a globular 125-amino-acid region (Fig. 5). If the α -helices were to interact in antiparallel, a finger-like structure would result, with a length of ~10 nm and a terminal globular domain (Fig. 5). Antiparallel coiled-coils have been identified in seryl-tRNA-synthetase¹⁹ and GreA²⁰, in both cases protruding from a globular domain. Such a structure is strikingly reminiscent of the

mysterious 'stalks' observed to extend from the globular heads of purified axonemal (length 11 nm)^{21,22} and cytoplasmic (length 8–18 nm)²³ dyneins reported by quick-freeze deep-etch or negative-staining techniques.

To examine the microtubule-binding region by ultrastructural means, a fragment encompassing amino acids 3,167–3,506 was expressed in insect cells using a baculovirus expression system. The recombinant polypeptide co-sedimented with microtubules, supporting a direct microtubule interaction (Fig. 4a). Examination of the recombinant polypeptide by deep-etch electron microscopy revealed two classes of particle (Fig. 4b). Most prominent were astral arrays of 53 ± 3 nm in diameter containing 12–18 radiating spokes. The spokes measured 12 ± 3 nm in length with 6 ± 2 nm globular swellings at their tips, virtually identical to the previously described dynein stalks^{21–23}. These radial aggregates probably result from nonspecific interactions at the fragment ends. Also observed in our preparations were short rods $23 \pm 3 \times 6 \pm 2$ nm. In some cases, a swelling appears to be present at one end. Whether these structures represent individual stalks thickened by the metal shadow, or aggregates of two or more stalks is uncertain. Cytoplasmic dynein stalks have been reported to self-associate in negative stained whole-molecule preparations²³.

We propose that the 346-amino-acid dynein heavy-chain fragment corresponds to the previously observed stalk^{21–23} and is composed of an anti-parallel coiled-coil. The 131-amino-acid subfragment, which alone is capable of interacting with microtubules (Figs 2, 3k, l), probably represents the bulbous stalk tip (Fig. 5) and is responsible for microtubule binding.

Of relevance to this model, electron microscopy (EM) of outer-arm dyneins in axonemes showed the dynein heads to be attached to the B-microtubules through fine fibres comparable in length to the stalks associated with individual dynein molecules^{21,22}. Furthermore, the globular dynein head did not make direct contact with the B-microtubule in either the presence or absence of ATP²², consistent with observations of dynein-decorated microtubules *in vitro*²⁴.

Interestingly, short deletion mutations of 7 and 10 amino acids in the first α -helix of *Chlamydomonas* outer-arm dynein β -heavy chain have been shown to suppress flagellar paralysis resulting from central-pair and radial-spoke defects²⁵. Such deletions should shorten the length of the extended stalk by one or one-and-a-half helical turns, respectively, without necessarily affecting microtubule binding. They do not seem to interfere with force

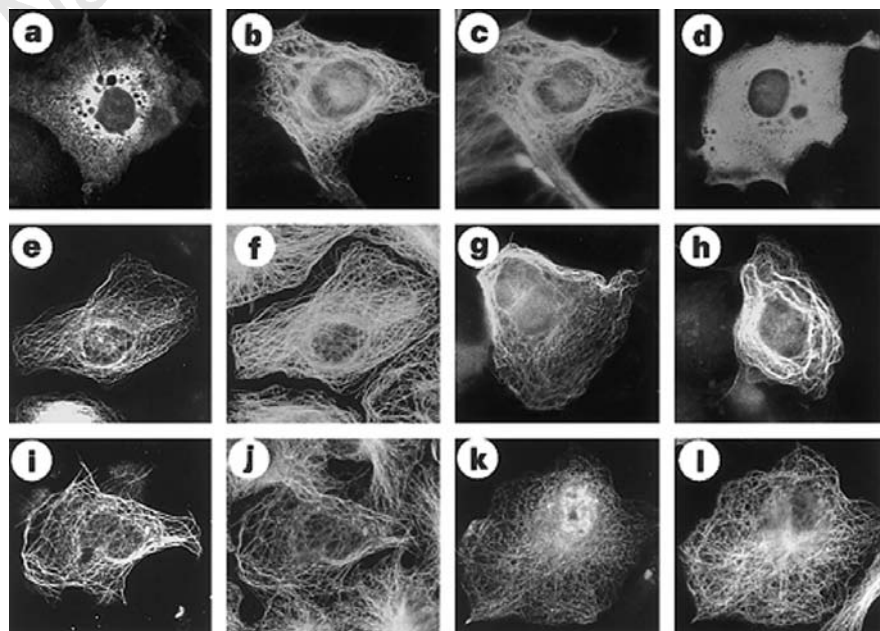


Figure 3 Co-distribution of mutant dynein heavy chain with microtubules. Immunofluorescence microscopy of cells transfected with **a**, full-length recombinant dynein heavy chain cDNA construct, and mutant constructs: **b, c**, K1910E; **d**, N1137; **e, f**, N1455; **g**, N3111; **h**, C3847; **i, j**, N3160/C3506; and **k, l**, N3276/C3407. Polyclonal anti-Flag antibody was used to detect recombinant protein in **a, b, d, g, h, i** and **k**, and anti-rat dynein heavy-chain antibody¹² was used in **e**. Monoclonal anti-tubulin antibody was used to visualize microtubules in **c, f, j** and **l**.

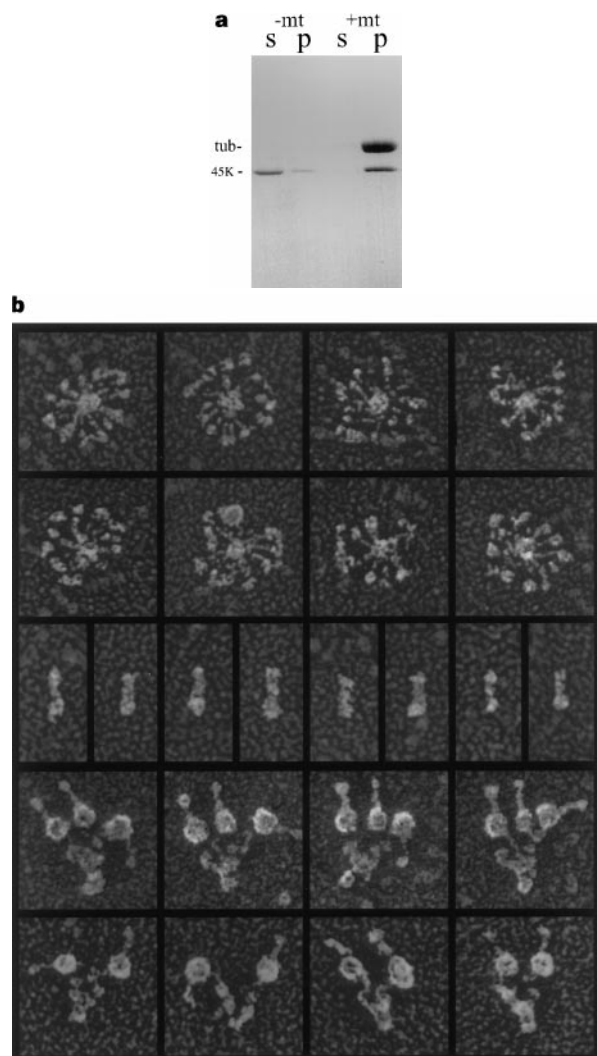


Figure 4 Microtubule-binding and electron microscopic analysis of recombinant microtubule binding domain. **a**, Co-sedimentation of purified recombinant polypeptide (45K) in the presence of microtubules (tub); s, Supernatant; p, pellet; mt, microtubules. Protein was visualized by Coomassie blue staining of a 12% polyacrylamide gel. **b**, Purified polypeptide was analysed by rotary shadow electron microscopy²⁷. Top two rows, astral arrays of individual stalks; third row, rods; fourth row, *Tetrahymena* flagellar outer-arm dynein, purified according to ref. 22; fifth row, cytoplasmic dynein purified according to ref. 28. Note the comparable size and morphology of stalks within radial arrays relative to those associated with flagellar dynein heads. All images were taken at an original magnification of 300,000x.

production in *in vitro* microtubule sliding assays²⁶. We speculate that the shorter stalks cause a slight decrease in intermicrotubule spacing and, therefore, the axonemal radius, which could in some way compensate for loss in regulation imparted by the radial spokes.

The large distance between the microtubule- and nucleotide-binding regions within the dynein heavy chain raises several questions about the mechanism of force production. The myosin actin-binding site and kinesin microtubule-binding site are both located within the globular head domains^{4–8}. However, in dynein, the globular head has an estimated diameter of 13 nm (ref. 22), much larger than the 4–5-nm tubulin subunit with which it interacts. Contact by an extended stalk might, therefore, be sterically advantageous.

Several possibilities can be envisaged for how force produced in the head might be transduced through an apparently rigid dynein stalk (Fig. 5). A conformational change in the dynein head could apply tension to the stalk and pull on the microtubule. Alternatively,

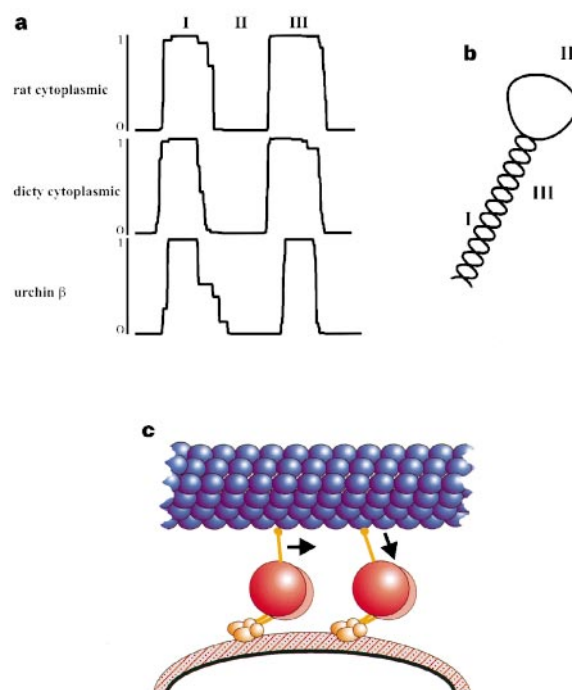


Figure 5 Structural and functional predictions for the dynein stalk. **a**, Coiled-coil analysis of microtubule binding region (residues 3,121–3,561) from the heavy chain of rat cytoplasmic dynein¹¹, and corresponding regions of (residues 3,202–3,642) *Dictyostelium discoideum* cytoplasmic dynein²⁹ and (residues 2,971–3,416) sea urchin axonemal β -dynein^{14,15}, as predicted using the NEWCOILS program³⁰. **b**, Proposed structure of dynein heavy-chain stalk showing predicted interaction of α -helices. **c**, Two models portraying the role of dynein stalks in motility. Diagram of cytoplasmic dynein molecules attached to a membranous vesicle at the bottom and to a microtubule at the top. Arrows indicate the stalk on the left exerting force towards the right, and the stalk on the right pulling the microtubule towards the right.

the stalk might rotate about a fulcrum located within the head, like a windshield wiper. We note that the angular rotation observed for the helical 'arm' of seryl-tRNA synthetase upon tRNA binding¹⁹ is comparable to our estimate of the angular rotation in the stalk required to move the dynein molecule from one tubulin subunit to the next.

How microtubule binding might be modulated during the cross-bridge cycle remains to be seen. A similar problem is posed by certain transmembrane receptors with helical membrane-spanning domains that transmit conformational information across the membrane. In the case of the dynein stalk, a twist or bend resulting from slight changes in the register or extent of interaction of its two α -helices might be involved.

A complete understanding of the dynein conformational cycle during motility must await further work. Nonetheless, our data indicate that components of the dynein motor domain are organized quite differently from those of either the kinesins or myosins, indicating that the mechanism for force production is different. □

Methods

Cloning and expression. Rat cytoplasmic dynein heavy-chain deletions and mutations were generated using a combination of polymerase chain reaction (PCR), linker, and restriction enzyme fragments ligated together in-frame. All heavy-chain fragments were tagged in-frame at the extreme N or C terminus with either the Flag (IBI) or c-Myc epitope by either PCR mutagenesis or insertion of DNA linkers encoding the tag. Constructs were prepared in the shuttle vector, pARK, a derivative of pBluescript (Stratagene), and subcloned into the *NotI* sites of the mammalian expression vector, pCMV β (Clontech). pCMV constructs were transiently transfected into COS-7 cells using

lipofectamine (Gibco BRL) according to the manufacturer's instructions. Cells were processed for VO_4 photocleavage assay or immunostaining 24–36 h after the start of transfection.

For expression of recombinant dynein microtubule-binding domain using baculovirus, a PCR fragment corresponding to residues 3,167–3,506 was cloned into the *Bam*HI and *Xba*I sites of the insect expression vector pFastBacHTb (BRL) containing an N-terminal 6-histidine tag. Recombinant baculovirus was obtained following the protocol for the Bac-To-Bac baculovirus expression system (BRL). $10 \mu\text{l}$ of second-amplification viral supernatant was used to infect 1×10^6 Hi5 insect cells (Invitrogen) and protein extracts were prepared three days after infection. Cells were suspended in lysis buffer (50 mM Tris, pH 8.5, containing 10 mM β -mercaptoethanol, AESF, aprotinin, leupeptin and TAME) and homogenized with a glass–Teflon homogenizer at 2,000 r.p.m. Extracts were then centrifuged at 4,000g at 4°C for 10 min, followed by 33,000g at 4°C for 30 min. The cleared extract was passed over a Ni^{2+} affinity column, which was then washed with 60 mM imidazole, 500 mM NaCl, 20 mM Tris, pH 7.9. Recombinant protein was eluted with 300 mM imidazole, 150 mM NaCl, 20 mM Tris, pH 7.9. Peak fractions were combined and dialysed into either Tris/KCl buffer (20 mM Tris, pH 7.6, 50 mM KCl, 5 mM MgCl_2 , 0.5 mM EDTA, 1 mM DTT) or PO_4/KCl buffer (10 mM KPO_4 , pH 7.5, 50 mM KCl, 5 mM MgCl_2 , 0.5 mM EDTA, 1 mM DTT).

Immunological methods. Antibodies used include the M2 mouse monoclonal anti-Flag antibody (IBI), a rabbit polyclonal anti-dynein heavy-chain antibody produced against the P-loop region of the rat cytoplasmic dynein heavy chain¹², and a monoclonal anti- α -tubulin antibody (Amersham). To produce polyclonal anti-epitope tag antibodies, rabbits were immunized with synthetic peptides corresponding to the c-Myc and Flag epitope tags. Immunoblotting was done as described¹², using the ECL reaction (Kirkegaard and Perry Laboratories) to visualize immunoreactive electrophoretic bands. For immunofluorescence microscopy, transfected cells were fixed using –20°C methanol for 9–12 min, processed for immunofluorescence staining, and viewed using a Zeiss Axiophot photomicroscope.

VO_4 -mediated photocleavage. Cell extracts were prepared by lysing transiently transfected cells on ice for 20 min in buffer containing 35 mM PIPES, pH 7.2, 5 mM MgCl_2 , 1 mM EGTA, 1 mM DTT, protease inhibitors (AESF, leupeptin, aprotinin and TAME) and 1% Nonidet-P40. Extracts were centrifuged at 4,000 r.p.m. for 10 min at 4°C to remove cell debris. ATP and VO_4 were added to 100 and 50 μM , respectively, and extracts were irradiated with ultraviolet light on ice for 90 min¹³.

In vitro microtubule-binding assays. Purified recombinant microtubule-binding domain (0.1 mg ml^{-1}) was mixed with 0.125 mg ml^{-1} of taxol-stabilized microtubules in PO_4/KCl buffer and incubated at 25°C for 45–60 min. Microtubules were then pelleted at 12,000g for 20 min at room temperature in a table-top microcentrifuge. Pellets were resuspended to volume, and equal volumes of supernatant and pellet were analysed using SDS 12% PAGE.

Electron microscopic imaging. In preparation for EM, proteins were absorbed to a suspension of ground mica flakes, followed by freeze-drying and platinum replication according to established procedures²⁷. This involved adding two drops of a suspension of finely ground mica flakes to 0.5 ml of a solution containing ~10 mg purified protein and allowing adsorption for 30 s. Mica flakes were then pelleted by gentle centrifugation, washed twice with 30 mM HEPES (pH 7.4), 70 mM KCl, 5 mM MgCl_2 and 3 mM EGTA, and then layered onto a slice of aldehyde-fixed rabbit lung for support during quick-freezing, which was accomplished by dropping the samples onto a liquid-helium-cooled copper block in a home-made device.

Quick-frozen mica flakes were freeze-fractured in a Balzer's freeze-etch machine and 'deep-etched' for 4 min at –100°C. The exposed proteins were rotary-replicated with ~2 nm of platinum. Replicas were cleaned of mica by floating them in concentrated hydrofluoric acid, washed in water, and picked up on 75-mesh formvar-coated microscope grids. Replicas were viewed in a JEOL transmission electron microscope and photographed in stereo using +10° of tilt with a eucentric side-entry goniometer stage. Representative molecules were chosen by video imaging of micrographs initially generated at 68,000 $\times$ and were transferred to a computer using a Targa 2000 A/D converter, enlarged to 300,000 $\times$, and placed in photo-montages with Adobe Photoshop.

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1. Gibbons, I. R. & Rowe, A. Dynein: a protein with adenosine triphosphatase activity from cilia. *Science*

- 149, 424–426 (1965).
2. Paschal, B. M. & Vallee, R. B. Retrograde transport by the microtubule associated protein MAP 1C. *Nature* **330**, 181–183 (1987).
3. Vallee, R. B. & Sheetz, M. P. Targeting of motor proteins. *Science* **271**, 1539–1544 (1996).
4. Kull, F. J., Sablin, E. P., Lau, R., Fletterick, R. J. & Vale, R. D. Crystal structure of the kinesin motor domain reveals a structural similarity to myosin. *Nature* **380**, 550–555 (1996).
5. Sablin, E. P., Kull, F. J., Cooke, R., Vale, R. D. & Fletterick, R. J. Crystal structure of the motor domain of the kinesin-related motor ncd. *Nature* **380**, 555–559 (1996).
6. Woehlke, G. *et al.* Microtubule interaction site of the kinesin motor. *Cell* **90**, 207–216 (1997).
7. Rayment, I. *et al.* The three-dimensional structure of myosin subfragment 1: a molecular motor. *Science* **261**, 50–58 (1993).
8. Ruppel, K. M. & Spudich, J. A. Structure–function analysis of the motor domain of myosin. *Annu. Rev. Cell Dev. Biol.* **12**, 543–573 (1996).
9. Porter, M. E. & Johnson, K. A. Dynein structure and function. *Annu. Rev. Cell Biol.* **5**, 119–151 (1989).
10. Holzbaur, E. L. F. & Vallee, R. B. Dyneins: molecular structure and cellular function. *Annu. Rev. Cell Biol.* **10**, 339–372 (1994).
11. Mikami, A., Paschal, B. M., Mazumdar, M. & Vallee, R. B. Molecular cloning of the retrograde transport motor cytoplasmic dynein (MAP 1C). *Neuron* **10**, 787–796 (1993).
12. Mazumdar, M., Mikami, A., Gee, M. A. & Vallee, R. B. In vitro motility from recombinant dynein heavy chain. *Proc. Natl Acad. Sci. USA* **93**, 6552–6556 (1996).
13. Gibbons, I. R. *et al.* Photosensitized cleavage of dynein heavy chains: cleavage at the "V1 site" by irradiation at 365 nm in the presence of ATP and vanadate. *J. Biol. Chem.* **262**, 2780–2786 (1987).
14. Gibbons, I. R., Gibbons, B. H., Moc, G. & Asai, D. J. Multiple nucleotide-binding sites in the sequence of dynein β heavy chain. *Nature* **352**, 640–643 (1991).
15. Ogawa, K. Four ATP binding sites in the midregion of the β -heavy chain of dynein. *Nature* **352**, 643–645 (1991).
16. Koonce, M. P. & Samso, M. Overexpression of cytoplasmic dynein's globular head causes a collapse of the interphase microtubule network in *Dictyostelium*. *Mol. Biol. Cell* **7**, 935–948 (1996).
17. Kanai, Y. *et al.* Expression of multiple tau isoforms and microtubule bundle formation in fibroblasts transfected with a single tau cDNA. *J. Cell Biol.* **109**, 1173–1184 (1989).
18. Mitchell, D. R. & Brown, K. S. Sequence analysis of the *Chlamydomonas* alpha and beta dynein heavy chain genes. *J. Cell Sci.* **107**, 635–644 (1994).
19. Biou, V., Yaremchuk, A., Tukalo, M. & Cusack, S. A crystal structure of *T. Thermophilus* seryl-tRNA synthetase complexed with tRNA^{Ser}. *Science* **263**, 1404–1410 (1994).
20. Stebbins, C. E. *et al.* Crystal structure of the GreA transcript cleavage factor from *Escherichia coli*. *Nature* **373**, 636–640 (1995).
21. Goodenough, U. W. & Heuser, J. E. Substructure of the outer dynein arm. *J. Cell Biol.* **95**, 798–815 (1982).
22. Goodenough, U. W. & Heuser, J. E. Structural comparison of purified dynein proteins with *in situ* dynein arms. *J. Mol. Biol.* **180**, 1083–1118 (1984).
23. Amos, L. A. Brain dynein crossbridges microtubules into bundles. *J. Cell Sci.* **93**, 19–28 (1989).
24. Haimo, L., Telzer, B. R. & Rosenbaum, J. L. Dynein binds to and crossbridges cytoplasmic microtubules. *Proc. Natl Acad. Sci. USA* **76**, 5759–5763 (1979).
25. Porter, M. E., Knott, J. A., Gardner, L. C., Mitchell, D. R. & Dutcher, S. K. Mutations in the SUP-PF-1 locus of *Chlamydomonas reinhardtii* identify a regulatory domain in the β -dynein heavy chain. *J. Cell Biol.* **126**, 1495–1507 (1994).
26. Smith, E. F. & Sale, W. S. Regulation of dynein-driven microtubule sliding by the radial spokes in flagella. *Science* **257**, 1557–1559 (1992).
27. Heuser, J. E. Procedure for freeze-drying molecules adsorbed to mica flakes. *J. Mol. Biol.* **169**, 155–195 (1983).
28. Steuer, E. R., Heuser, J. E. & Sheetz, M. P. Cytoplasmic dynein and ciliary outer arm dynein: a structural comparison. *Cell Motil. Cytoskel.* **11**, 200–201 (1988).
29. Koonce, M. P., Grissom, P. M. & McIntosh, J. R. Dynein from *Dictyostelium*: Primary structure comparisons of a cytoplasmic motor enzyme and flagellar dynein. *J. Cell Biol.* **119**, 1597–1604 (1992).
30. Lupas, A., Van Dyke, M. & Stock, J. Predicting coiled coils from protein sequences. *Science* **252**, 1162–1164 (1991).

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Structure of the proteasome activator REG α (PA28 α)

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The specificity of the 20S proteasome, which degrades many intracellular proteins, is regulated by protein complexes that bind to one or both ends of the cylindrical proteasome structure^{1–5}. One of these regulatory complexes, the 11S regulator (known as REG or PA28), stimulates proteasome peptidase activity^{6,7} and enhances the production of antigenic peptides for presentation by class I molecules of the major histocompatibility complex (MHC)^{8,9}. The three REG subunits that have been identified, REG α , REG β and REG γ (also known as the Ki antigen),

share extensive sequence similarity, apart from a highly variable internal segment of 17–34 residues which may confer subunit-specific properties¹⁰. REG α and REG β preferentially form a heteromeric complex¹¹, although purified REG α forms a heptamer in solution¹² and has biochemical properties similar to the heteromeric REG α /REG β complex^{13,14}. We have now determined the crystal structure of human recombinant REG α at 2.8 Å resolution. The heptameric barrel-shaped assembly contains a central channel that has an opening of 20 Å diameter at one end and another of 30 Å diameter at the presumed proteasome-binding surface. The binding of REG probably causes conformational changes that open a pore in the proteasome α -subunits through which substrates and products can pass.

The human recombinant REG α monomer has a relative molecular mass of 28,700 (M_r 28.7K) and is predominantly helical, with four long α -helices each containing 33–45 residues (Figs 1, 2a). Helices 2, 3 and 4 pack against each other along their entire lengths. A 45-degree kink at Pro 34 allows the carboxy-terminal end of helix 1 to pack against helices 2 and 4; the amino-terminal end projects away from the rest of the monomer. The sequence between helices 1 and 2 includes a disordered 39-residue loop which contains the subunit-specific sequence motifs that distinguish the different REG subunits^{10,11} (Fig. 1). The two ends of this loop visible in electron density maps are adjacent to each other at the top of Fig. 2a and b, thus constraining the subunit-specific sequences to the vicinity of the opening of 20 Å diameter to the central channel through the recombinant REG α heptamer. Interactions between helices of the

same monomer are predominantly hydrophobic, with a few buried polar associations, such as a buried salt bridge between Arg 181 of helix 3 and Asp 205 of helix 4. This charge interaction seems to orient Arg 181 appropriately for an additional salt bridge with Asp 196 of the adjacent molecule in the REG α heptamer.

Monomers make extensive interactions with their neighbours in the heptamer, with monomer–monomer interfaces burying 1,750 Å² of solvent-accessible surface area from each molecule. Unlike the predominantly hydrophobic interactions between helices within a monomer, intermolecular interactions are a mix of both polar and hydrophobic associations. The most extensive intermolecular interaction is a parallel association between helix 2 of one monomer and helix 4 of the neighbouring molecule. The heptamer is further stabilized by the N-terminal portion of helix 1 and the preceding extended residues, which project away from the monomer structure to interact with helices 1 and 4 of the neighbouring molecule. Consequently, the N-terminal portions of helix 1 from the seven molecules have the appearance of a belt around the outside of the heptamer. As the biochemical properties of our human recombinant REG α closely resemble those of the mixed REG α /REG β complex purified from red blood cells^{13,14}, it is likely that the heteromeric REG α /REG β complex is also a heptamer (perhaps with a random distribution of REG α and REG β subunits), although others have concluded that the REG α /REG β complex is a hexamer^{15,16}.

Mutagenic data implicate at least five residues in the loop from Arg 141 to Gly 149 in binding and activation of the proteasome

Table 1 Crystallographic statistics

	Native	Thimerosal
Wavelength (Å)	0.980	1.5418
Resolution (Å)	40.0–2.80	30.0–3.38
High resolution (Å)	(2.85–2.80)	(3.46–3.38)
No. of unique reflections	32,245	21,612
Completeness (%)	98.4 (95)	74.7 (72.6)
$\langle I/\sigma(I) \rangle$	13.3 (2.9)	6.3 (3.4)
Redundancy*	8.9 (2.7)	5.0 (1.5)
R_{sym} (%)†	8.7 (24.1)	9.6 (30.6)
Mosaicity (°)	0.5	0.79

Native data were collected on a MAR imaging plate detector on beamline 9-1 of the Stanford Synchrotron Radiation Laboratory (SSRL). The derivative data were collected on an R-Axis imaging plate detector with a rotating anode source. Values in parentheses refer to data in the high-resolution shell.

* Redundancy is the ratio of observed/unique structure factor amplitudes.

† $R_{\text{sym}} = 100 \times \sum_i \sum_j |I_i - I_j| / \sum_i \sum_j I_i$.

Table 2 Refinement statistics

Resolution (Å)	10.0–2.8
R -factor (%)*	24.8
R -free (%)†	28.9
R.m.s.d. (bonds) (Å)	0.020
R.m.s.d. (angles) (°)	1.670
No. of residues (total)	200 (249)
$\langle B \rangle$ (Å ²)	28.1
No. of ϕ/ψ angles (%): most favoured‡	91.2
additional allowed	7.9
other	0.9

* R -factor = $100 \times \sum_i |F_p(\text{obs}) - F_p(\text{calc})| / \sum_i F_p(\text{obs})$.

† R -free = R -factor for a randomly selected subset (5%) of the data that were not used for minimization of the crystallographic residual.

‡ Stereochemistry was analysed with PROCHECK²⁹.

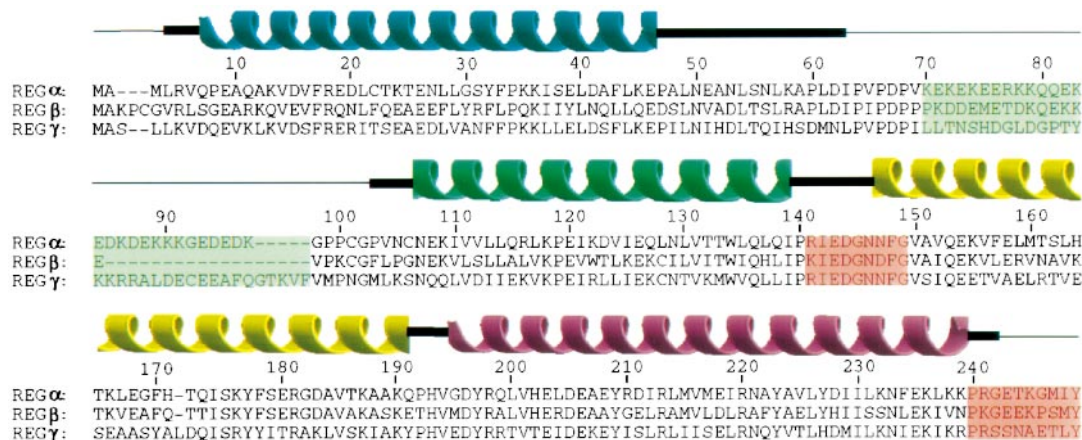


Figure 1 REG α secondary structure and sequence alignment with human REG β and REG γ . Helices are indicated as follows: helix 1 (blue; residues 7–46), helix 2 (green; 107–139), helix 3 (yellow; 147–191), and helix 4 (magenta; 195–239). Helix 1 is kinked by ~45° at Pro 34, and helix 4 has a severe ~65° kink at Phe 234. The 48 residues that lack electron density (1–3, 64–102, 243–249) have been omitted from

the model and are indicated by a thin line. Analysis by SDS-PAGE indicates that the disordered loop has not been cleaved in the crystal. The subunit-specific variable-insert residues (70–97) are shown against a green background. Sequences implicated in binding and activation of the proteasome (141–149, 240–249) are shown on a red background.

(Z.Z. *et al.*, manuscript submitted). We also designate the last 10 residues of REG as a binding/activation sequence because Pro 240 is critical for proteasome binding, with Met 247 and the conserved C-terminal residue Tyr 249 also being important for this interaction (Z.Z. *et al.*, manuscript submitted). Others have reported similar findings for Tyr 249 (ref. 17). These activation and binding sequences are coloured red in Figs 1, 2a–c. Although the C-terminal six residues of REG α are disordered in the crystal, the last ordered residues are immediately adjacent to the activation loop. Thus, residues that bind the proteasome are clustered together on one face of the REG α heptamer, indicating that this is the proteasome-binding surface (Fig. 2c).

Proteasomes comprise 28 subunits stacked in four rings of seven subunits each, with central rings of β -subunits that contain the proteolytic active sites and outer rings of α -subunits that bind regulatory complexes^{18,19}. Substrates reach the catalytic centres by passing through the central opening along the seven-fold axis of the α -subunits²⁰. A plausible model for the REG/proteasome complex, which is consistent with an electron microscopy study⁴, can be constructed by alignment of the REG α and proteasome seven-fold axes (Fig. 3). Substrates presumably enter and/or products leave this complex through the central REG α pore, which has a diameter of 30 Å at the proteasome-binding end but narrows to 20 Å at the opposite end (Fig. 3, top). This restriction implies that if substrates enter through the REG α pore, they must already be unfolded when

they first enter the proteasome/REG complex through the 20-Å opening. The pore is lined by residues from helix 3, almost all of which are polar. Of these side chains, four are positively charged (Lys 155, Lys 176, Lys 187 and Lys 190) and five are negatively charged (Glu 154, Glu 158, Glu 168, Glu 180 and Asp 183).

Residues implicated in proteasome binding form a ring on the human recombinant REG α heptamer of 40–55 Å diameter. In the model for the REG/proteasome complex shown in Fig. 3, these REG residues sit approximately over residues 13–34 of the proteasome α -subunits. This modelling exercise used coordinates of the proteasome from the archaeon *Thermoplasma acidophilum*, for which the first 12 residues of the proteasome α -subunits are disordered¹⁸. The recently determined yeast proteasome coordinates are not yet available, although the two proteasome structures closely resemble each other: an important difference is that the N-terminal residues of the α -subunits are ordered and completely block access to the central channel in the yeast proteasome structure¹⁹. This provides a mechanism by which nonspecific hydrolysis is prevented by the eukaryotic proteasome, and indicates that binding of REG must cause a conformational change in N-terminal sequences of the proteasome α -subunits to facilitate substrate access to or product release from the proteasome active sites. The conformational change presumably involves at least the first 12 or so residues of the proteasome α -subunits¹⁸, although it is not clear whether these residues contact REG directly. In an active complex the proteasome

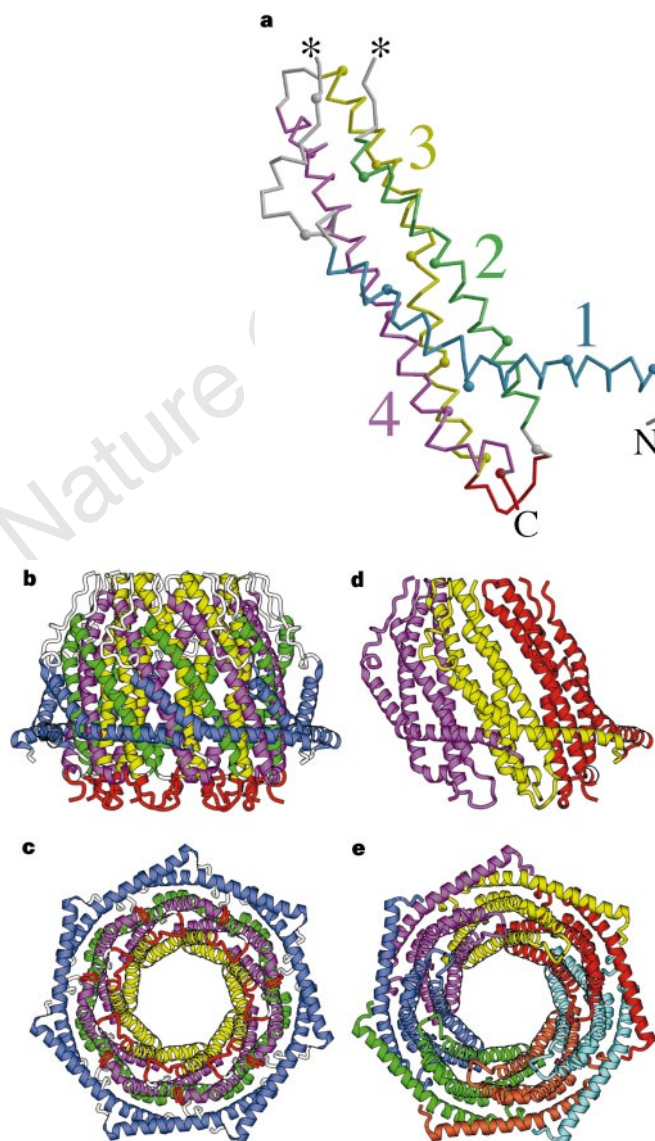


Figure 2 Structure of REG α . **a**, Stereo view of a monomer C α representation coloured by secondary structural element. Every tenth residue is indicated by a sphere. Disordered residues are not modelled in any of the figures. Colour coding is the same as in Fig. 1. The ends of the disordered 39-residue loop are indicated by asterisks. **b**, The heptamer viewed with the seven-fold axis vertical, as in **a**, with the monomer nearest the viewer in the same orientation as in **a**. **c**, Heptamer viewed along the seven-fold axis, from underneath that shown in **b**. **d**, The three subunits nearest the viewer are shown in the same orientation as in **b**. As shown for the central cyan subunit, each monomer contacts just two other subunits. **e**, Heptamer coloured by individual monomers and viewed from the same direction as in **c**. This figure was made using MOLSCRIPT³⁰.

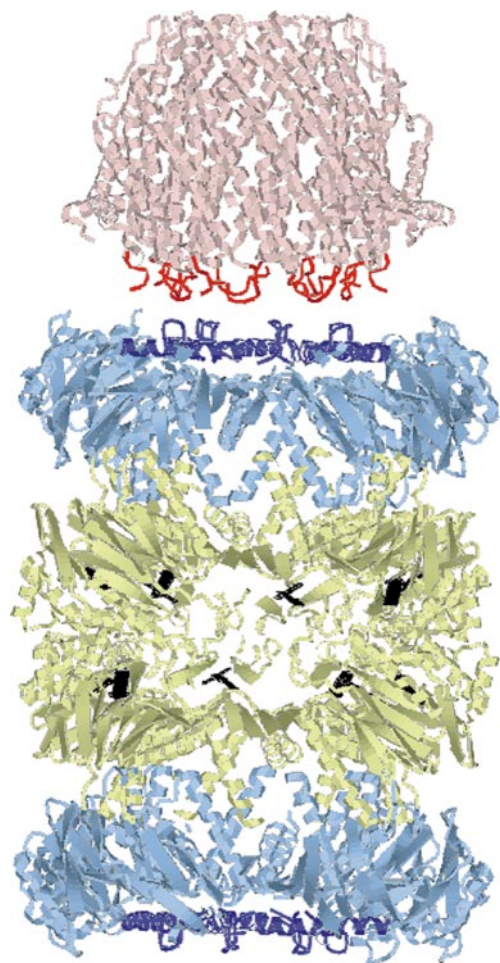


Figure 3 Model for the interaction of REG with the proteasome. Seven-fold axes are aligned for REG α (top; pink) and the 20S proteasome (below; α - and β -subunits are shown in light blue and light green respectively). Segments of REG implicated in proteasome binding and activation are shown in red. Segments of proteasome that are most likely to contact REG are in darker blue (residues 13–34). The N-terminal 12 residues of the α -subunits are disordered in the crystal structure shown here of the proteasome from *T. acidophilum*¹⁸; in the yeast proteasome, residues are ordered and block the central pore through the α -subunits¹⁹. Additional proteasome residues may contact the disordered C-terminal residues of REG. Residues near the proteasome active sites are shown in black. In order to see the location of active sites in the central chamber, this representation of the proteasome has been cut away at the face towards the viewer.

N-terminal sequences may be bound up in the central pore of the REG heptamer, although this is unlikely as it would probably block the route of substrate entry to the proteasome. It seems more likely that the N-terminal sequences move away from the centre of the REG/proteasome complex, possibly as a large-scale displacement of the entire first helix of each proteasome α -subunit.

Biochemical studies with short model peptide substrates reveal that REG activates proteasomes as much as 200-fold against some peptides, whereas activity against other similar peptides is almost unchanged^{6,7,11}. An explanation for this selective activation could be that REG presents an opening that allows selective access (or egress) of one tri- or tetrapeptide but not another, although this is unlikely as the crystal structure does not suggest a mechanism for such discrimination. Also, REG is not likely to function by binding and delivering a substrate to the proteasome because, unlike the 19S cap which associates with 20S proteasomes to form the 26S

protease^{1,21,22}, REG does not have an ATPase activity to promote active transport. We favour a model in which, in addition to the proposed change in the α -subunits already discussed, binding of REG also causes a conformational change in the proteasome β -subunits: this would explain the dramatic range in degree of activation towards peptides of similar size by selectively enhancing hydrolysis of different sequences at the catalytic centres. Because the proteasome catalytic sites are some 60 Å from the putative REG binding surface (Fig. 3), we propose that binding of REG induces a long-range conformational change that is transmitted to the β -subunits through the proteasome α -subunits. This is consistent with observations that REG acts as a positive allosteric effector of proteasome activity⁷ and that it alters the cleavage preference of the proteasome^{9,11}.

The REG α -specific insert sequence, which is rich in lysine and glutamic acid residues, forms a 'KEKE' motif that has been proposed to mediate protein–protein interactions²³. The disordered 39-residue loop that contains this sequence is located at the top edge of the heptamer, about 60 Å distant from the putative proteasome binding surface, so the variable insert sequences probably do not directly mediate proteasome binding or activation. Indeed, we find that deletion of the REG α insert sequence does not affect peptidase activity (Z.Z. *et al.*, manuscript submitted). Instead, the structure is consistent with a role for the subunit-specific sequences in defining intracellular localization, perhaps targeting activated proteasomes to components of the membrane of the endoplasmic reticulum²³. The structure is also consistent with a role for the variable sequences in binding to chaperone complexes which could present unfolded proteins to the central pore. An attractive feature of this second model is that it provides a mechanism for the degradation of proteins, as opposed to unstructured peptides, by harnessing the ability of chaperones to generate unfolded conformations for entry into the proteasome's central chamber. □

Methods

Crystallization and data collection. Recombinant, human REG α was expressed, purified and crystallized as described¹². Briefly, crystals of REG α were grown at 4 °C in 4- μ l sitting drops containing a 1:1 mixture of protein solution (10 mg ml⁻¹ REG α in 10 mM MOPS pH 7.2, 0.2 mM EDTA, 1 mM DTT) and reservoir solution (12% PEG 6K, 100 mM Na-MOPS, pH 7.2, 2 M NaCl, 0.9 mM ZnCl₂). The space group is C2 ($a = 162.6$ Å, $b = 134.3$ Å, $c = 116.2$ Å, $\beta = 90.6^\circ$), with one heptamer per asymmetric unit. Before data collection at 100K, crystals were cryoprotected in a solution of 20% glycerol, 13.5% PEG 6000, 2 M NaCl, 100 mM Na-MOPS, pH 7.1. Crystals were then suspended in a rayon loop and cooled by plunging into liquid nitrogen.

Native data were collected from a single crystal at the Stanford Synchrotron Radiation Laboratory (SSRL) beamline 9-1 using a MAR imaging-plate detector. The isomorphous heavy-atom derivative was obtained by soaking a native crystal for 45 min at 4 °C in reservoir solution made up with 1 mM thimerasol. Derivative data were collected from a single crystal on an RAXIS II imaging plate detector with a rotating-anode source. Data were processed with DENZO and SCALEPACK²⁴ (Table 1).

Structure determination and refinement. Most crystallographic computations used programs from the CCP4 suite²⁵. The mercury sites of the heavy-atom derivative were identified from isomorphous difference Patterson and Fourier functions, and refined with the program MLPHARE to a figure of merit of 0.35 at 5.0 Å resolution. The SIR map calculated at 5 Å resolution showed a clear distinction between solvent and protein regions, and the arrangement of the 14 mercury heavy-atom sites in two rings at either end of the REG α heptamer defined initial operators for non-crystallographic symmetry (NCS) averaging of the SIR map²⁶. The averaged 5-Å SIR map showed tubes of density corresponding to α -helices. A mask was constructed around one REG α monomer, and phase refinement/extension was performed through averaging, histogram shifting and solvent flattening with the program DM. The resulting map showed well defined density into which a model was built using the program O (ref. 27). Five percent of the observed amplitudes were reserved for cross-validation and refinement with NCS restraints was performed using

X-PLOR²⁸. As model building and refinement progressed, the monomer mask and NCS operators were updated and unbiased experimental maps recalculated. No sigma cut was applied during refinement (Table 2). Solvent molecules were not included in the model.

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- Coux, O., Tanaka, K. & Goldberg, A. L. Structure and functions of the 20S and 26S proteasomes. *Annu. Rev. Biochem.* **65**, 801–847 (1996).
- Hoffman, L. & Rechsteiner, M. in *Current Topics in Cellular Regulation* (eds Stadtman, E. R. & Chock, P. B.) 1–32 (Academic, San Diego, 1996).
- Peters, J.-M., Cejka, Z., Harris, J. R., Kleinschmidt, J. A. & Baumeister, W. Structural features of the 26S proteasome complex. *J. Mol. Biol.* **234**, 932–937 (1993).
- Gray, C. W., Slaughter, C. A. & DeMartino, G. N. PA28 activator protein forms regulatory caps on proteasome stacked rings. *J. Mol. Biol.* **236**, 7–15 (1994).
- Peters, J.-M. Proteasomes: protein degradation machines of the cell. *Trends Biochem. Sci.* **19**, 377–382 (1994).
- Ma, C.-P., Slaughter, C. A. & DeMartino, G. N. Identification, purification and characterization of a protein activator (PA28) of the 20S proteasome (macropain). *J. Biol. Chem.* **267**, 10515–10523 (1992).
- Dubiel, W., Pratt, G., Ferrell, K. & Rechsteiner, M. Purification of an 11S regulator of the multicatalytic protease. *J. Biol. Chem.* **267**, 22369–22377 (1992).
- Groettrup, M. et al. A role for the proteasome regulator PA28 α in antigen presentation. *Nature* **381**, 166–168 (1996).
- Dick, T. P. et al. Coordinated dual cleavage induced by the proteasome regulator PA28 leads to dominant MHC ligands. *Cell* **86**, 253–262 (1996).
- Ahn, J. Y. et al. Primary structures of two homologous subunits of PA28, a γ -interferon-inducible protein activator of the 20S proteasome. *FEBS Lett.* **366**, 37–42 (1995).
- Realini, C. et al. Characterization of recombinant REG α , REG β and REG γ proteasome activators. *J. Biol. Chem.* **272**, 25483–25492 (1997).
- Johnston, S. C., Whitby, F. W., Realini, C., Rechsteiner, M. & Hill, C. P. The proteasome 11S regulator subunit REG γ (PA28 α) is a heptamer. *Prot. Sci.* **6**, 2469–2473 (1997).
- Realini, C., Dubiel, W., Pratt, G., Ferrell, K. & Rechsteiner, M. Molecular cloning and expression of a gamma-interferon inducible activator of the multicatalytic protease. *J. Biol. Chem.* **269**, 20727–20732 (1994).
- Ustrell, V., Realini, C., Pratt, G. & Rechsteiner, M. Human lymphoblast and erythrocyte multicatalytic proteases: differential peptidase activities and responses to the 11S regulator. *FEBS Lett.* **376**, 155–158 (1995).
- Ahn, K. et al. *In vivo* characterization of the proteasome regulator PA28. *J. Biol. Chem.* **271**, 18237–18242 (1996).
- Song, X. et al. A model for the quaternary structure of the proteasome activator PA28. *J. Biol. Chem.* **271**, 26410–26417 (1996).
- Ma, C.-P., Willy, P. J., Slaughter, C. A. & DeMartino, G. N. PA28, an activator of the 20S proteasome, is inactivated by proteolytic modification at its carboxyl terminus. *J. Biol. Chem.* **268**, 22514–22519 (1993).
- Löwe, J. et al. Crystal structure of the 20S proteasome from the archaeon *T. acidophilum* at 3.4 Å resolution. *Science* **268**, 533–539 (1995).
- Groll, M. et al. Structure of 20S proteasome from yeast at 2.4 Å resolution. *Nature* **386**, 463–471 (1997).
- Wenzel, T. & Baumeister, W. Conformational constraints in protein degradation by the 20S proteasome. *Nature Struct. Biol.* **2**, 199–204 (1995).
- Hochstrasser, M. Ubiquitin, proteasomes, and the regulation of intracellular protein degradation. *Curr. Opin. Cell Biol.* **7**, 215–223 (1995).
- Jentsch, S. & Schlenker, S. Selective protein degradation: a journey's end within the proteasome. *Cell* **82**, 881–884 (1995).
- Realini, C., Rogers, S. W. & Rechsteiner, M. KEKE motifs: Proposed roles in protein-protein association and presentation of peptides by MHC class I receptors. *FEBS Lett.* **348**, 109–113 (1994).
- Otwinowski, Z. in *Data Collection and Processing* (eds Sawyer, L., Isaacs, N. & Bailey, S.) 56–62 (SERC Daresbury Laboratory, Warrington, UK, 1993).
- CCP4. The CCP4 suite: programs for protein crystallography. *Acta Crystallogr. D* **50**, 760–763 (1994).
- Kleywegt, G. J. & Jones, T. A. in *From First Map to Final Model* (eds Bailey, S., Hubbard, R. & Waller, D.) 59–66 (SERC Daresbury Laboratory, Warrington, UK, 1994).
- Jones, T. A., Zou, J.-Y., Cowan, S. W. & Kjeldgaard, M. Improved methods for building protein models in electron density maps and location of errors in these models. *Acta Crystallogr. A* **47**, 110–119 (1991).

- Brünger, A. T. *X-PLOR Version 3.843, A System for X-ray Crystallography and NMR* (Yale University, New Haven, CT, 1996).
- Laskowski, R. A., MacArthur, M. W., Moss, D. S. & Thornton, J. M. PROCHECK: a program to check the stereochemical quality of protein structures. *J. Appl. Crystallogr.* **26**, 283–291 (1993).
- Kraulis, P. J. Molscript: a program to produce both detailed and schematic plots of protein structures. *J. Appl. Crystallogr.* **24**, 946–950 (1991).

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Correspondence and requests for materials should be addressed to C.P.H. (e-mail: chris@mscc.med.utah.edu). Coordinates (lavo) have been deposited in the Brookhaven Protein Data Bank.

correction

CTL induction by a tumour-associated antigen octapeptide derived from a murine lung carcinoma

Ofer Mandelboim, Gideon Berke, Mati Fridkin, Michael Feldman, Miriam Eisenstein & Lea Eisenbach

Nature **369**, 67–71 (1994)

In this Letter, we reported that the octapeptides Mut1 and 2 (52 FEQNTAQP and A59, respectively), thought to originate from a mutated form of the gap-junction protein connexin 37 with a Cys → Gln mutation in position 3 of the peptide, are shared tumour-specific antigenic epitope(s) of the C57BL/6 Lewis lung carcinoma 3LL clones D122 and K^b39.5, and that they are capable of inducing tumour-specific CTL, tumour immunoprotection, and immunotherapy of established lung metastasis (see also ref. 1). More recent experiments performed in the laboratory of one of us (G.B.) now call into question this finding and conclusions. The experimental details and results will be published elsewhere²; a preprint is available on request. □

- Mandelboim, O. et al. Regression of established murine carcinoma metastases following vaccination with tumor associated antigen peptide. *Nature Med.* **1**, 1179–1183 (1995).
- Rosen, D., Brokenthal, K. & Berke, G. An appraisal of the tumor immunoprotective and therapeutic activities of the octapeptides Mut1 and 2 from the mouse Lewis lung carcinoma 3LL. *J. Immunol.*, submitted.

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Imaging is not everything

This week's section focuses on cell biology tools – items viewed include imaging and microscopy systems, an intracellular injector, media and media additives, oligosaccharide selections and systems for plant cell biology.

MetaGFP 3.0

From Universal Imaging

Software for the collection and analysis of images from typical GFP experiments

MetaGFP was designed to collect single and time-series images of fluorescence and transmitted light from GFP or similar fluorescent proteins and probes. The program automates image collection by controlling the illumination with filter wheels, shutters and monochromators, as well as managing the camera functions (video, integrating and cooled digital CCD). The program overlays the fluorescent images with the transmitted light images into publication-quality, 24-bit colour or monochrome images, and allows interactive manipulation of the colour balance. The resulting colour images are displayed using a new movie feature or by exporting the images into any standard presentation graphics software. Version 3.0 takes advantage of the 32-bit capabilities of Windows95 to provide faster operation, long descriptive file names and advanced printing options.

Reader Enquiry No. 100

Spot

From Diagnostic Instruments

A cooled CCD colour digital camera designed specifically for use with light microscopes

The camera uses three-shot digital technology to derive true, non-interpolated red, green and blue values from the 1.4-million pixel array. It is said to provide a viable alternative to film photography and a much higher image capture resolution than is achieved by an analog camera/framegrabber combination. Spot software is designed to



Spot the microscope software.

make digital photomicrography easier to perform without the need for advanced imaging training. The camera is said to be free from vibration and utilizes a fast focus window for near real-time focusing. Peltier cooling is used to cool the chip to -12°C (at 25°C , ambient room temperature). According to the manufacturer, this cooled CCD provides brilliant low-light-level colour images for applications, such as fluorescence, polarized-light, dark-field and high-magnification work. Spot software features automatic exposure or user-defined, image-acquisition settings. Post-picture editing, image annotation and a database archival system are all included in the software. A TWAIN driver is provided, as is a driver that is currently available for use with the 32-bit version of Image Pro Plus.

Reader Enquiry No. 101

PCM2000

From Nikon

A compact confocal microscopy system designed as an alternative to multi-user systems

The system is designed so that individual laboratories can now afford a fully featured, confocal system. It has been designed to be smaller and more compact, with the scanner module attaching directly to the microscope. The result is a modular system that can be placed on any suitable laboratory bench. Images are obtained by raster scanning through a microscope with a focused point of laser light. No alignment procedures are required for the scanner module or laser — the scanner module can be adapted to upright or inverted microscopes. Moreover, the PCM2000 is said to provide high-resolution images of elusive phenomena, such as calcium uptake by neurons during nerve impulses. Scanning, image manipulation and analysis are controlled through the system's menu driven software.

Reader Enquiry No. 102

AnalySIS Docu

From Olympus

A new searchable, image archiving system for microscopy

Image archiving is the main function of this system, however, image processing, interactive measurement and process automation, through the use of macros, are also included. As part of a suite of modular archiving and analysis software, the system can be scaled up to the full professional image analysis system called analySIS Pro. As the storage of digital images can be mem-



Digital archiving with Olympus' analySIS system.

ory intensive, the analySIS system offers multiple-volume capability, using magneto-optical (MO) disks. The use of MO disks for image archiving is said to have improved data security, access speed and price performance. AnalySIS Docu provides for distribution and handling of image data to several MO disks, thus allowing storage and management of nearly unlimited quantities of data. Information describing examined specimens, whether calibration and numeric data, comments or other references, can be stored along with the digital images. Query and routines are created easily by logically linking individual search terms from a mouse-selected term lists. This allows complex search routines to be performed when necessary. The system offers password protection and imaging features, such as length and surface area measurement, linear filters and changes of image geometry.

Reader Enquiry No. 103

Laboratory equipment

Mastercycler thermal cyclers

From Eppendorf

The Mastercycler personal and the Mastercycler gradient are characterized by a high degree of flexibility in PCR experiments

An important feature of these units is the use of 'personal cards', which can store up to ten programs, thus extending programs capacity. The cards have the same format as a cheque card and increase the 100-position memory of both thermal cyclers, facilitating data transfer between the devices and eliminating the need for a computer link-up. Users can store their own, individually developed programs on a personal card, calling them up on Mastercycler as needed. The device's software and hardware also allow for connection to a PC or printer. Other benefits include the unique universal block for 0.2-ml and 0.5-ml test tubes and microtitre plates, precise block homogeneity and high-temperature control speeds, as well as the gradient function featured on Mastercycler gradient.

Reader Enquiry No. 104



WPI's PM2000 cellular perfusion system.

PM2000 pressure injector

From World Precision Instruments

Programmable four-channel pressure injector

Designed for intracellular injection and extracellular perfusion, this system may be used for injecting different molecular agents directly into cells, including labelled dyes, genes, RNA, DNA, proteins and cell organelles. It can perform perfusions of up to 15 different combinations from four different agents and drugs into a cell or a group of cells. The PM2000 is also useful for intracytoplasmic sperm injection and cell electrophysiology. A micro-controller has been combined with precise pneumatic components to permit simultaneous performance of such tasks as injection, capillary action balancing, clear up or suction. Customized action sequences can be programmed with up to 16 sequences and 32 programmable steps in each sequence.

Reader Enquiry No. 105

Incubators

From Thelco

High-performance laboratory incubators

Thelco incubators are said to offer more standard features and precise temperature control for a wide range of demanding incubation applications. These include bacterial growth, microbiological determinations, tissue culturing, histochemical procedures, haematological testing, crystallization studies, pharmaceutical stability testing, and more. There are six models and four sizes from which to choose, including mechanical or gravity-heat convection. Chamber capacities range from 40–157.5 litres and have temperature ranges from 5–70 °C, as well as precise uniformity (0.25 °C), sensitivity (0.1 °C) and stability (0.2 °C).

Reader Enquiry No. 106

Media scrutiny

Vitacell growth medium

From ATCC

Cell culture media and serum

The manufacturer states that quality control tests performed on the media and serum include thorough quantification of clone-forming efficiencies and sequential growth curve studies with multiple adherent and suspension culture. The initial offering includes five liquid media formula-

tions: RPMI-1640 medium, Dulbecco's modified Eagle's medium, minimum essential medium Eagle, F-12K medium and Iscove's modified Dulbecco's medium. They will be available in 500-ml plastic bottles with glutamine already added for convenience. Fetal bovine serum will also be available in 500-ml bottles.

Reader Enquiry No. 107

Pluronic F-68

From Mediatech

Part of a line of insect tissue culture reagents

Pluronic F-68 is a non-ionic detergent that protects cells in suspension cultures from shearing caused by bubbling, stirring and shaking. The molecule is believed to interact with the cell membrane forming a hydration sheath that protects the cell from shearing. Insectagro Pluronic F-68 is available as a powder in 100-g size or as a 10 per cent solution ($\times 100$) in packs of 6 $\times$ 100-ml glass bottles.

Reader Enquiry No. 108

Photosynthetic opportunity

PLC4-FL leaf chamber

From ADC

New lightweight leaf chamber that combines gas exchange and chlorophyll fluorescence analysis

Typical applications for the system include investigating the effects of environmental changes, such as air pollution and elevated carbon dioxide levels, and use in crop management and yield improvement schemes. Based on the standard broad-leaf chamber, the unit is compatible with ADC's LCA-4 photosynthesis and transpiration measurement system and the ADC:OSI 5FL pulse modulated fluorometer. Boundary layer resistance and temperature gradients are minimized within the leaf chamber. Electronic sensors measure chamber and direct leaf temperature, between -5 and 50 °C. Chamber temperature is maintained



The Chamber: ADC's lightweight leaf chamber.

within ± 0.3 °C and direct leaf temperature within ± 0.5 °C. Ambient PAR is also measured and the instrument has been designed to minimize the amount of shading caused by the fibre-optic cable linking the ADC:OSI 5FL to the measurement site.

Reader Enquiry No. 109

Bio-Fence

From Applied Photosynthetics

For the production of photosynthetic microorganisms, such as algae and bacteria

This is a closed photobioreactor that has been designed to produce maximum treatment efficiencies in temperate climatic conditions. Available in three ranges with capacities up to 25 litres per fence, 250 litres per fence unit (connecting in parallel up to 2,500 litres), and 2,500 litres per fence unit (connecting in parallel to larger scale). The system can be used for long periods without culture crash occurring, says the manufacturer. It is also said to be very simple to manage and can be customized to suit individual needs.

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Fencing in photomicrobes with Bio-Fence.

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CI-500

From Cottenham Instruments & Materials
A photosynthesis measurements system

The CI-500 is a complete system that includes a display, a keypad, a computer, data memory, a CO₂/H₂O gas analyser, a flow control system and a battery — all contained in a single hand-held case. The system has all the components needed to measure photosynthesis, transpiration, stomatal conductance, internal CO₂ and, optionally, chlorophyll fluorescence. There are 12 interchangeable chambers for different types of leaves and for both open and closed systems.

Reader Enquiry No. 111

Biochemical reagents

E_{max} immunoassays

From Promega Neurosciences

New ELISA systems for the sensitive and specific detection of BDNF (brain derived neurotrophic factor) and soluble gp130

The ELISA format allows accurate quantification in the picogram per millilitre range. Two sizes are available: a package for 400 determinations plus standards or a package for 160 determinations plus standards. Both offer optimized reagents and protocols, so that the user can configure plates in any desired format. Promega also offers E_{max} immunoassay systems for GDNF, NGF, NT-3, and TGFβ1 and β2.

Reader Enquiry No. 112

Calbindin D-28K polyclonal antibody

From Chemicon

A polyclonal antibody to calbindin is a calcium binding protein found in subpopulations of neurons

This binding protein is found in the CNS, retina, sensory ganglia, cerebellar Purkinje cells, substantia nigra and some peripheral sensory organs. Through its calcium buffering capacity, calbindin D-28K is believed to act as an important regulator of neuronal activity. Unlike other polyclonal antibodies to calbindin D-28K, this new rabbit antibody does not crossreact with the 55 per cent homologous calretinin protein, as may be found in western blots. According to the manufacturer, it has been used successfully for immunohistochemistry on human, rat and mouse brain.

Reader Enquiry No. 113

Oligosaccharides

From Sigma

A comprehensive 16-page product guide features bioactive oligosaccharides

Intended for researchers involved in cell biology, immunology, drug design, micro-

biology, biochemistry, analytical glycobiology and protein chemistry, Sigma's *Complex Carbohydrates Product Guide* lists 96 different oligosaccharides. For ease of use, the guide groups products first by their constituent sugar subunits and then alphabetically by common name within the group. Also included with each listing is a crossreference indicating the major applications for each product, as well as available package sizes. Where appropriate, elemental analysis and enzymatic assays for substrate suitability are also performed.

Reader Enquiry No. 114

Enzyme selection guide

From Oxford GlycoSciences

An informative enzyme selection guide in poster format

The guide provides information that may

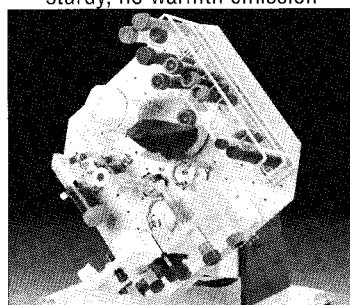
be necessary for selecting the correct enzymes for analysis of glycoproteins and other glycoconjugates. The enzymes are used in a variety of applications, including glycoconjugate structural analyses, functional studies, glycosaminoglycan research, GPI anchor detection, carbohydrate remodelling and synthesis. The guide contains a comprehensive list of available enzymes, divided into endo-, exo- and other glycosidases for easy selection, with details of their specificities, incubation conditions and typical applications, together with an indexed reference section.

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